THE METABOLISM OF *PARAMECIUM CAUDATUM*

I. RESPIRATION

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Metabolic studies with protozoa have been carried out mainly by determining the O_2 consumption with and without the presence of added compounds which might serve as substrates for the animals' needs. The use of protozoa for such investigations presents many difficulties. For example, nearly all of these organisms can be reared only in the presence of other living matter and in most cases the quantity of protozoa obtained is very small. In one instance (*Tetrahymena geleii*), these difficulties have been overcome and reliable nutritional (Kidder and Dewey, 1946) and enzymatic (Seaman, 1952) studies have been made. With other organisms, however (e.g. the widely-used *Paramecium caudatum*) methods of culture are not so advanced and the difficulties arising from this deficiency are partly responsible for the confused and incomplete state of our knowledge regarding the biochemistry of these types.

Most of the work on *P. caudatum* and related species of this genus has been directed to the question of the cyanide-stability of the respiration; this work has been briefly reviewed in a previous paper (Humphrey and Humphrey, 1948). The present work was commenced in 1949 and seeks to outline the respiratory characteristics of the organism as shown by changes in the O_2 consumption brought about by the presence of various organic compounds which might act as substrates for, or inhibitors of, respiration. We have also endeavoured to relate our experiments to the well-being of the animal and have paid particular attention to the motility, morphology, etc., of the organisms at the conclusion of each experiment. Failure to do this has lead to many of the conflicting reports in the literature. Recently, van Grembergen and Reynaerts-de Pont (1952) have made a careful study of the effect of many compounds on the O_2 consumption of *P. caudatum*.

MATERIALS AND METHODS.

P. caudatum was cultured as previously described (Humphrey and Humphrey, 1948), The cultures were grown in 4 litre Haffkine flasks containing 2 litres of boiled medium.

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Histological dyestuffs were obtained from G. T. Gurr (London). Cytochrome *c* was isolated by the method of Keilin and Hartree (1937) and its concentration determined spectrophotometrically by reduction with $Na_2S_2O_4$. Oxalacetic acid was prepared according to Wohl and Oesterlin (1901), *iso*-citric acid according to Nelson (1930) and *cis*- and *trans*-aconitic acids by the methods of Bruce (1937) and Malachowski and Maslowski (1928). Other compounds were obtained from commercial sources.

In general, the methods used were those previously described, measurements being carried out in 0·5 p.c. Osterhout solution at 27° C.

The production of ammonia was determined by placing the reactants in the outer chamber of a Conway vessel whose inner compartment contained 1 ml. 0·02 N H_2SO_4; the lid was put in place and incubation allowed to proceed. At the end of the experimental period 1 ml. saturated K_2CO_3 was added to the outer chamber and the NH_3 liberated was estimated by direct Nesslerization.

Oxygen consumption was estimated polarographically by the two-potential method (Petering and Daniels, 1938) or manometrically with Warburg flasks of 10 ml. capacity. The polarograph was constructed so that three cells (10 ml. lipless beakers) could be used simultaneously. Mercury was allowed to drop through capillaries consisting of clinical thermometer tubing; this gave a mercury flow of 15–20 drops each minute. Current flow at 0·1 and 1·0 volts was recorded on a 450 ohm Cambridge spot galvanometer. The two methods were used to ensure that the results obtained were not artefacts produced by the particular technique adopted; usually both methods were used.

Respiration and NH_3 production were calculated on the basis of 10^4 organisms for an hourly period as Q_{O_2} (μl.O_2 consumed) or Q_{NH_3} (μl.NH_3 produced). In all cases the number of animals used gave results well above the sensitivity of the method being used. The organisms were examined microscopically after each experiment and unless otherwise stated, motility and morphology were normal.

During the four years of the investigation the animals showed an increasing sensitivity to the salt concentration of the suspending medium. This meant that when using substrates and inhibitors alone or together (each to give a final concentration of 0·01 M in most cases), the composition of the basic suspending medium (0·5 p.c. Osterhout-Sorenson phosphate) had to be varied in the following manner. Initially, and for the greater part of the time, 0·03 M phosphate was used, then 0·01 M and finally, 0·003 M. Care was taken to ensure that, when osmotically-sensitive organisms were used, the inhibitors and substrates were replaced in the controls by Osterhout solution of similar molarity. It was not necessary to allow for the difference in activity of equimolar solution of Osterhout (mainly NaCl), and reagents which were non-ionized or ionized into more than two components.

RESULTS.

Effect of centrifugation. The organisms were isolated from the culture medium by the method of Geddes and Humphrey (1951). The strain of *P. caudatum* used suffered no damage on centrifugation; growth curves of centrifuged animals were very similar to those from controls (Fig. 1). Staining of wet preparations was carried out by adding a drop of suspension to a drop of glycerol; the staining fluid was then gently mixed with the preparation and the mixture examined under high power. Neutral red, methylene blue, Janus green B, methyl green and iodine were used to demonstrate structures such as nuclei, vacuoles, cilia and mitochondria; in no instance was there apparent any damage to, or displacement of, these structures.

Effect of mercury. The experimental period for polarographic measurement of oxygen consumption was usually 1 hour. At the end of this time the animals were swimming as vigorously as control specimens left in media containing no mercury; there was also no change observable in the structure of the organisms. The effect of mercury on the growth rate of cultures was also investigated; some variation in the shape of the curves was observed but there was little change in the maximum concentration of organisms (Fig. 1).

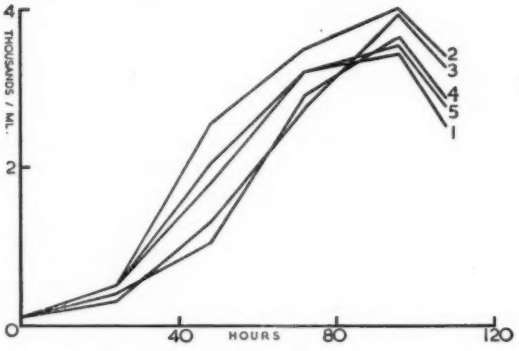

Fig. 1.

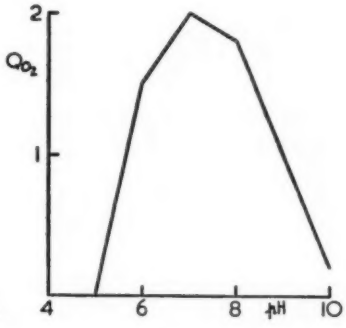

Fig. 2.

Fig. 1. Growth curves. 1. Control, 2. centrifuged animals for inoculum, 3. as 1 but Hg. present during growth, 4. as 1 but inoculum was with animals left 1 hour in contact with Hg., 5. as 4 but centrifuged animals used.

Fig. 2. Effect of pH on Q_{O_2}.

Effect of pH on respiration. This was determined in the presence of 0·03 M Sorenson phosphate buffer (Fig. 2). Below pH 5, clumping and death of the organisms occurred; the optimum value for respiration varied between 6·6 and 7·0 in different experiments.

Age of organisms and respiration. The effect of age on the oxygen consumption is shown in Fig. 3; the experimental fluid contained 0·03 M phosphate, pH = 7·0. It can be seen that the younger the organism, the greater the amount of O_2 consumed by each individual.

Effect of oxygen concentration. The pO_2 of 0·5 p.c. Osterhout solution was reduced by boiling and cooling out of contact with air. By such means the amount of oxygen dissolved in 1 p.c. Osterhout solution could be brought to half its normal value; this lowered the Q_{O_2} considerably (Fig 4). Microscopically the organisms were unaffected by keeping for the experimental period (1 hour) in either these oxygen-deficient media or in 1 p.c. Osterhout solution made oxygen-free by the passage of nitrogen which had been passed over Cu turnings at 400° C.

Effect of concentration of suspension. Using organisms of the same age and 1 p.c. Osterhout solution saturated with air, the Q_{O_2} was found to be constant when the number of animals was varied between 1·2 and $5·0 \times 10^4$/ml. (Table 1).

TABLE 1.

Respiration and concentration of organisms.

Concentration 10^4/ml.	$Q_{O_2}/10^4$ organisms
1·2	1·2
1·8	1·1
2·3	1·1
5·0	1·1

Respiration in the presence of added substrate. The effect of fatty acids, sugars, amino-acids and di- and tri-carboxylic acids on the respiration of the animals was tested with cultures of low and high osmotic sensitivity. The concentration of the compounds being tested was either 0·01 or 0·02 M. The increase in respiration observed varied from time to time but certain generalizations have been made. For example, with regard to fatty acids, acetate and propionate could almost double the O_2 uptake whereas butyrate, valerate and caprylate gave smaller increases. Carbohydrates were not very active in this regard, glucose being the most effective with almost 50 p.c. stimulation, followed by mannose, lactose, fructose, sucrose, xylose, galactose, arabinose and maltose With organic acids, *cis*-aconitate, *α*-ketoglutarate and *iso*-citrate could double or treble the O_2 uptake; lactate was not so effective and then came pyruvate, oxalacetate, fumarate, succinate, malate and citrate in that order. No amino-acid was able to double the O_2 uptake; the most effective was glycine, followed by L-arginine, L-glutamate, glutamine, DL-threonine, DL-phenylalanine, L-proline, L-aspartate, DL-alanine, asparagine, DL-serine, norleucine, L-leucine, L-histidine, DL-isoleucine, L-tryptophane, L-lysine and DL-valine.

Production of ammonia. In the absence of any added substrate, the organisms produce a small amount of ammonia; this formation can be increased by the presence of amino-acids. The experiments were carried out in the presence of 1 p.c. Osterhout solution containing 0·02 M or 0·003 M phosphate (pH = 6·8) and 0·01 M amino-acid. In the absence of added amino-acid the values for Q_{NH_3} lay between 0·10 and 0·50/10^4 organisms. Asparagine was the most effective compound used, giving 570 p.c. of the control with water; then followed DL-alanine (410 p.c.), glutamine (400 p.c.), DL-serine (340 p.c.), L-hydroxyproline (260 p.c.), DL-methionine (240 p.c.), L-aspartic acid (240 p.c.), norleucine (220 p.c.), L-histidine (210 p.c.), DL-isoleucine (190 p.c.), L-proline (190 p.c.), L-arginine (180 p.c.), L-leucine (160 p.c.), L-lysine (150 p.c.), L-cysteine (140 p.c.), L-tryptophane (140 p.c.), DL-valine (130 p.c.), L-glutamic acid (120 p.c.) and glycine (110 p.c.).

DISCUSSION.

Effect of pH.

The variation in respiration brought about by using media of different acidity, can be caused by alterations in the enzymic activity, the transport of metabolites throughout the protoplasm of the organism or indirectly by changes in the functioning of various systems such as cilia, contractile vacuoles, etc. Gaw (1936) studied the contraction of vacuoles in several species of *Paramecium* as a function of pH; in the range of 5·8 to 8·9, 7·0 was shown to be optimal. Humphrey and Humphrey (1948) showed that the O_2 consumption of homogenates of *P. caudatum* was markedly affected by pH; the range 6·6 to 7·6 proved to be optimal, activity falling off sharply on the alkaline side and rather more slowly on the acid side. Using whole cells of *P. caudatum*, van Grembergen and Reynaerts-de Pont (1952) found hardly any pH effect between 6·5 and 8·0 and a slight diminution down to 4·9. Our own results show a definite maximum around 7·0 but between 6·5 and 8·0 there is only a small change.

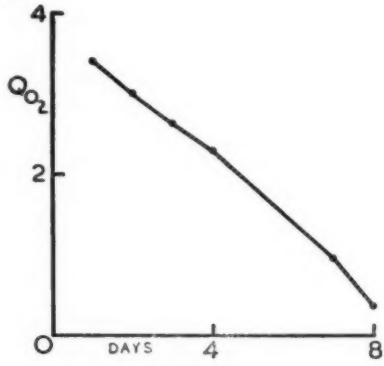

Fig. 3.

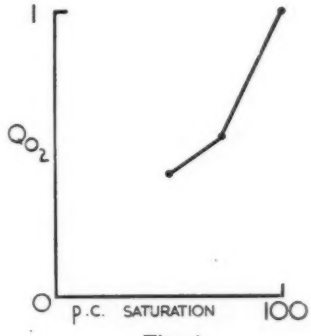

Fig. 4.

Fig. 3. Effect of age of culture on Q_{O_2}.

Fig. 4. Effect of initial pO_2 (as p.c. saturation with air) on Q_{O_2}.

Age of culture.

The falling off in intensity of O_2 uptake with increasing age of the animals (Fig. 3) could be due to senescent changes in the organisms themselves or changes brought about by variations in pO_2, pH and rH of the medium. Further, since the animals are dependent for their nutrition on the associated bacteria and moulds, changes in these populations may play a part. No differences in metabolic activity were found between 2, 3 and 4-day cultures. With organisms which could be grown in sterile media (*Chilomonas paramecium*), Hutchens (1941) found that both the O_2 consumption and the respiratory quotient varied inversely with the age of the culture.

Effect of oxygen supply.

The various conflicting reports in the literature concerning the effects of alterations in pO_2 on the respiration and well-being of *P. caudatum* have been critically examined by von Brand (1946). Some authors claim that the organisms can live for only a few seconds in the absence of O_2 while other workers have managed to keep them alive for several days. The results we obtained with regard to O_2 consumption are contrary to most of the previous reports, including that of van Grembergen and Reynaerts-de Pont 1952) who found the respiratory intensity unaffected in the range used (5–100 p.c. O_2).

Intensity of respiration and concentration of animals.

Because many investigators have used faulty technique, the majority of the values given in the literature for the endogenous Q_{O_2} of protozoa are not thoroughly reliable. The values for *P. caudatum* have been tabulated by several workers (e.g. Jahn, 1941; van Grembergen and Reynaerts-de Pont, 1952) but these Tables are misleading unless accompanied by a critical evaluation of the experimental methods. Choosing only values which seem based on proper methods, the Q_{O_2} of homogenates can be taken as 1–4 and of whole cells as 1–40, most of the figures for whole cells lying in the range 1–10. The large variation in the Q_{O_2} for whole cells can be explained as due to differences in age, culture medium, strain of organism, preparation of organism, the method used for measurement of O_2 uptake and individual differences. Most recently, Scholander *et al.* (1952) have developed a very sensitive type of capillary respirometer which uses a micro-Cartesian diver to record pressure; by these means single cells of *P. caudatum* were found to vary from each other in respiratory intensity from 200–1,000 $\mu\mu$l per hour (equivalent to a Q_{O_2} of 2–10 per 10^4 organisms). Our own figures for animals 2–4 days old, show a Q_{O_2} from 1–4 and these values are not dependent upon the concentration of the suspension.

Effect of substrates.

The results show that many compounds can increase the O_2 consumption, especially some lower fatty acids and some members of the citric acid cycle. Carbohydrates and amino-acids are also effective in this regard. Further, cytochhrome (0·02 mM.) was able to stimulate endogenous respiration by about 30 p.c., and could also increase the effectiveness of succinate, e.g. in a particular experiment cytochrome stimulated by 31 p.c., succinate by 43 p.c., and both together by 87 p.c. Methylene blue (0·1 mM.) increased the endogenous respiration by amounts varying from 50–300 p.c. When a compound does bring about an increase in O_2 uptake, we can conclude that the compound can enter into the metabolic reactions of the animal either catalytically

or as a utilizable substrate or perhaps that the respiration is stimulated by some non-specific action as a result of which ciliary movement, protoplasmic streaming or some other energy-requiring process is initiated or increased in intensity. Because we did not observe any change in the morphology and motility of the organisms after these experiments we conclude that the compounds acted as metabolites. Where no increase in O_2 uptake occurs, we cannot state that the compound does not function in metabolism, because rates of permeability and co-factor supply might be limiting factors.

The most important contribution on the question of substrate stimulation of metabolism in protozoa is the series of papers by Seaman relating to *Tetrahymena geleii*. In addition to other investigations, Seaman (1949) studied the effect of members of the citric acid cycle on the O_2 consumption; he showed that fumarate, succinate, malate, α-ketoglutarate and oxalacetate could each double the O_2 uptake whereas citrate had no effect. The only careful study reported in the literature of these matters with *P. caudatum* is that of van Grembergen and Reynaerts-de Pont (1952); their findings differ in a number of respects from those reported in the present investigation. These workers found marked stimulation of O_2 uptake with succinate, somewhat less with carbohydrates or pyruvate, only a small increase with malate and an increase with citrate only after an initial lag of 30–120 minutes; no change was observable in the presence of methylene blue. These discrepancies cannot be traced to variations or faults in technique but might rather be attributed to differences in the strains of animals used. Interpreting all the results obtained on *P. caudatum* in the light of modern biochemical ideas on metabolism, the operation of a citric acid cycle seems quite probable; further work on substrate utilization and the action of inhibitors is the next step in reaching a more definite conclusion.

Production of ammonia.

Using experimental periods of 12 hours, Emery (1928) found that *P. caudatum* could utilize many amino-acids with the production of NH_3; the endogenous Q_{NH_3} was $1{\cdot}7/10^4$ organisms and this value was increased by nearly 300 p.c. in the presence of suitable amino-acids. Autolysis and some bacterial action are probably responsible for the high values reported. No other figures could be found in the literature although Boell (1946) states that *P. calkinsi* produces "large quantities of ammonia."

Specht (1935), working with *Spirostomum ambiguum* (a much larger animal) obtained a Q_{NH_3} of $21/10^4$ organisms and observed that to get a true value of the Q_{O_2} ($= 26$) acid must be present in the side bulb of the Warburg flask to absorb the NH_3 evolved. No such effect of acid on the Q_{O_2} was observed in our experiments which showed a Q_{NH_3} of about one-tenth the value of the Q_{O_2}; the increases obtained in the presence of amino-acids confirm Emery's results, showing that active amino-acid metabolism can occur.

SUMMARY.

The oxygen consumption of *P. caudatum* was examined using the polarograph and Warburg manometers. Similar results were obtained, mercury having no effect on the growth or well-being of the animals.

The concentration of the experimental suspension did not affect the Q_{O_2} but young animals consumed more O_2 than old ones. The O_2 uptake was decreased as the pO_2 diminished.

Lower fatty acids and some members of the citric acid cycle were very effective in increasing the O_2 uptake. Carbohydrates, amino-acids and other members of the citric acid cycle also showed a stimulating effect, as did cytochrome and methylene blue.

The organisms produce a small quantity of ammonia and this production is increased by amounts up to 500 p.c. in the presence of amino-acids or their amides.

The results are discussed and it is concluded that strain differences probably account for the discrepancies between the findings of many workers who have made careful investigations. Present results seem to indicate that a citric acid cycle is operative in this animal.

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THE METABOLISM OF *PARAMECIUM CAUDATUM*

II. THE EFFECT OF RESPIRATORY INHIBITORS

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The use of compounds which inhibit the O_2 consumption of living cells has often given an indication of the presence of certain enzymic systems in those cells. This type of experimentation has proved to be a very powerful tool in the discovery and elucidation of the metabolic pathways which organisms use for energy production and other physiological reactions. In those cases where the living cells are whole animals such as protozoa, information of a different type can be gained about the action of the inhibitors from that obtained when experiments are carried out on cells and tissue preparations from higher animals. With the protozoa, effects on O_2 consumption can be related to effects on motility, morphology and other aspects of the animals' physiological well-being. Conversely, however, more care has to be taken when working with whole animals since the effects observed may be due to action upon a number of physiological systems and not solely upon some particular metabolic reaction or sequence of reactions.

Questions of this nature have been discussed in a previous paper (Holland and Humphrey, 1953) and the discussion will now be extended by considering the action of respiratory inhibitors.

Cyanide and azide are important members of this class of compounds and they have been used, with valuable results, to elucidate the rôle of cytochrome oxidase and the pathways of electron transfer in tissue preparations from the higher animals. The use of these two reagents in experiments on *Paramecium caudatum* has led to an extensive literature; much of it is of doubtful value and only recently has careful experimentation been employed. This has been reviewed briefly by Humphrey and Humphrey (1948) and with the claims of Boell (1946) about the *activating* effect of azide, the centre of interest has moved from cyanide to azide. Further, van Grembergen and Reynaerts-de Pont (1952) have tried the effect of some other agents; in this present work, several compounds have been used and their effects have been studied not only on O_2 consumption but also on the physiological integrity of the organisms.

[1] This investigation was supported by apparatus grants from the Research Committee and Cancer Research Committee, University of Sydney.

METHODS.

The methods used were those previously described (Holland and Humphrey, 1953). In addition, mixed suspensions of bacteria and moulds were prepared by filtering a wild culture of *P. caudatum* through sintered glass to remove the protozoa and then inoculating into fresh 0·1 p.c. vegemite in 0·5 p.c. Osterhout solution. The mixture was maintained at 28° C. and 5 ml. of this culture was added each day to every 100 ml. of protozoan culture on which growth curves in the presence of inhibitors were being determined.

Where cyanide was used in manometric experiments, the precautions advised by Robbie (1946) were always adopted. As previously, both the polarograph and Warburg manometers were used in the experiments.

RESULTS.

Changes in form and motility.

Table 1 shows the changes in the animals after contact with certain respiratory inhibitors. The inhibitor solutions were neutralized before use and the observations recorded are those made after 1 hour. With no inhibitor present or in the presence of 0·01 M NaCl, the organisms retained their normal state and swam very vigorously. Centrifuged animals reacted in a fashion similar to those from a wild culture; the presence of metallic mercury in the experimental or control vessels gave no change in the effects observed.

TABLE 1.

Effect of inhibitors on activity.

Compound	Concentration (mM.)	Effect on animals
arsenite	10	all died
	1	slower
	0·1	slightly slower
azide	10	larger, some died
	1	larger, slower, darting
	0·1	none
cyanide	10	slightly slower; somewhat enlarged
	1	slightly slower
	0·1	none
fluoride	0·1	none
malonate	20	none
selenite	10	slower, a few died
	1	slightly slower
	0·1	slightly slower
trans-aconitate	10	none
urethane	10	a few died, some developed blisters
	1	none died, some developed blisters

The effect of cyanide on animals which had been starved (left for 24 hours in 1 p.c. Osterhout solution) or which had been cultured in 0·1 mM. KCN, was the same as that observed with normal cultures.

Amino-acids were able to exert a protective effect against the action of azide. In these experiments 1 mM. azide and 10 mM. amino-acids (glycine, serine, alanine and aspartic acid) were used. During the 2 hours' observation, the organisms in media containing azide and an amino-acid showed greater activity and slightly less swelling but still displayed "darting movements" (a typical azide effect). The animals in media containing azide but no amino-acid, were very slow, somewhat bloated and continuously revolving.

Effect of inhibitors on growth.

The action of inhibitors on the growth of the organisms was tested by determining the maximum concentration of animals obtained and also the time required to produce that concentration. Because the compounds used also reduced the growth of the bacteria and moulds on which the animals fed, some experiments were carried out in which extra bacteria and moulds were added. The results of three sets of experiments are presented in Table 2.

TABLE 2.

Inhibitors and growth.

Inhibitor	Extra food	Maximum number/ml.	Time in hours
— — —	—	4,200	94
Cyanide, 10 mM.	—	0	—
Cyanide, 1 mM.	—	2,000	94
Cyanide, 0·1 mM	—	4,400	94
Azide, 10 mM.	—	0	—
Azide, 1 mM.	—	0	—
Azide, 0·1 mM.	—	0	—
— — —	+	3,200	72
Cyanide, 10 mM.	+	0	—
Cyanide, 1 mM.	+	3,000	72
Cyanide, 0·1 mM.	+	2,800	72
Azide, 10 mM.	+	0	—
Azide, 1 mM.	+	0	—
Azide, 0·1 mM.	+	1,000	94
— — —	+	5,500	95
Fluoride, 1 mM.	+	5,200	95
Urethane, 1 mM.	+	4,500	70
Arsenite, 1 mM.	+	0	—
Selenite, 1 mM.	+	0	—

Endogenous respiration.

Table 3 gives an outline of the effect of a number of compounds on the O_2 uptake in the absence of added substrate (cf. Table 1 for the effect on the physiological state).

TABLE 3.

Inhibitors and endogenous respiration.

Compound (mM.)	p.c. inhibition
Arsenate, 0·1	12
Arsenite, 0·1	60
Azide, 10	100
Azide, 1·0	90
Azide, 0·1	32
Cyanide, 10	100
Cyanide, 1	33
Cyanide, 0·1	30
Fluoride, 1·0	30
Fluoride, 0·1	10
Malonate, 20	31
Selenite, 0·1	10
Trans-aconitate, 10	25
Urethane, 10	32
Urethane, 1·0	4
Urethane, 0·1	—5

Some of the experiments with cyanide were carried out using starved animals and animals cultured in 0·1 mM. cyanide. With starved animals, 1 mM. cyanide inhibited 100 p.c. and 0·1 mM. 30 p.c. The corresponding figures for unstarved cyanide-grown animals were 15 and 6 p.c. The strains referred to in the previous paper (Holland and Humphrey, 1953) as osmotically sensitive gave smaller inhibitions than those noted in Table 3 for our first and main strain. In fact the third strain (tolerating 0·003 M phosphate) showed up to 41 p.c. increase of O_2 consumption in the presence of 1·0 mM. cyanide. This strain also gave up to 42 p.c. increase with 0·1 mM. azide. Unless otherwise stated, the results given are for the first osmotically resistant strain.

Inhibitors and respiration in the presence of substrates.

When inhibitors are added to systems with an appreciable endogenous respiration, the effect of the inhibitor on the respiration due to substrate must be calculated by allowing for the inhibition of the endogenous respiration.

e.g. let $E = Q_{O_2}$ (endogenous);
$S = Q_{O_2}$ (in the presence of added substrate);
$I = Q_{O_2}$ (E plus inhibitor);
$SI = Q_{O_2}$ (S plus inhibitor).
Then, inhibition of substrate respiration

$$= 100 \times \frac{(S\text{-}E) - (SI\text{-}I)}{(S\text{-}E)} \text{ p.c.}$$

This assumes that the endogenous and substrate respirations proceed without interaction. Table 4 gives results obtained with glucose as the substrate, in experiments using both the polarograph and Warburg manometers.

TABLE 4.

Inhibitors and glucose respiration.

The final concentration of glucose was 10 mM.

Inhibitor (mM.)	*E*	*S*	*I*	*SI*	p.c. inhibition
Arsenite, 0·1	2·38	3·77	1·65	3·25	—15
Azide, 0·1	1·50	2·35	1·00	1·24	72
Cyanide, 0·1	1·50	2·21	1·05	1·41	49
Fluoride, 0·1	2·20	2·64	1·98	2·25	39
Malonate, 20	1·50	2·21	1·05	1·52	34
Selenite, 0·1	1·45	2·50	1·32	1·60	74

TABLE 5.

Inhibitors and fatty acids.

Both the polaropraph and Warburg manometers were used.

Inhibitor (mM.)	Substrate (10 mM.)	*E*	*S*	*I*	*SI*	p.c. inhibition
Arsenite, 0·1	Acetate	1·46	4·50	0·58	0·88	90
Azide, 0·1	Acetate	1·46	4·50	1·00	1·43	86
Cyanide, 0·1	Acetate	2·13	4·96	1·49	1·69	93
Malonate, 20	Acetate	1·87	4·04	1·10	3·82	—26
Selenite, 0·1	Acetate	1·46	4·50	1·31	1·48	94
Urethane, 1·0	Acetate	1·76	2·86	1·84	2·48	42
Urethane, 0·1	Acetate	1·16	1·72	1·25	1·89	—15
Azide, 0·1	Propionate	2·00	4·55	1·67	4·90	—27
Cyanide, 0·1	Propionate	2·00	4·50	1·69	4·86	—27

Similar experiments were carried out with fatty acids (Table 5), amino-acids (Table 6) and organic acids (Table 7).

The third strain of the organism (showing increased O_2 consumption in the presence of cyanide and azide) gave further stimulation in the presence of these "inhibitors" and fatty acids, e.g. with cyanide and acetate, $E = 2{\cdot}53$, $S = 3{\cdot}55$, $I = 3{\cdot}47$ and $SI = 4{\cdot}49$. *Trans*-aconitate reacted similarly, activating both the endogenous and fatty acid respirations.

TABLE 6.

Inhibitors and amino-acids.

Both the polarograph and Warburg manometers were used.

Inhibitor	Substrate (10 mM.)	*E*	*S*	*I*	*SI*	p.c. inhibition
Azide, 1 mM.	Alanine	1·30	2·11	0·13	1·19	—31
Azide, 1 mM.	Aspartic acid	1·28	2·25	0·10	2·02	—98
Azide, 1 mM.	Glycine	1·06	2·07	0·12	2·23	—111
Azide, 1 mM.	Serine	1·20	1·59	0·13	1·11	—165
Cyanide, 1 mM.	Alanine	1·30	2·11	0·86	2·25	—72
Cyanide, 1 mM.	Aspartic acid	1·00	1·48	0·67	1·22	—15
Cyanide, 1 mM.	Glycine	1·42	2·66	0·96	2·82	—50
Cyanide, 1 mM.	Serine	1·22	1·80	0·80	1·54	—28

Thus, when O_2 uptake was diminished, the organisms could use amino-acids on an increased scale. To check that the amino-acids were being actually utilized, the NH_3 content of the experimental fluid from the polarographic cells was determined. These figures showed that in most of the experiments, cyanide and azide caused about a 50 p.c. rise in both the endogenous and amino-acid ammoniogenesis; in the remaining experiments, either no change or a slight drop in NH_3 production was noted.

TABLE 7.

Inhibitors and organic acids.

Both the polarograph and Warburg manometers were used.

Inhibitor	Substrate (10 mM.)	*E*	*S*	*I*	*SI*	p.c. inhibition
Azide, 0·1 mM.	pyruvate	2·08	2·40	1·98	2·33	—9
Azide, 0·1 mM.	citrate	2·30	3·10	1·85	2·54	14
Azide, 0·1 mM.	*iso*-citrate	1·26	1·73	0·87	1·18	34
Azide. 0·1 mM.	α-ketoglutarate	1·50	3·94	1·05	4·20	—29
Azide, 0·1 mM.	succinate	2·28	3·25	2·03	2·97	3
Azide, 0·1 mM.	fumarate	1·41	2·07	1·29	1·96	—2
Azide, 0·1 mM.	malate	2·28	3·07	2·03	2·82	0
Cyanide, 1 mM.	pyruvate	1·72	3·06	1·02	1·28	80
Cyanide, 1 mM.	citrate	1·75	2·27	1·30	1·51	60
Cyanide, 1 mM.	*iso*-citrate	1·93	2·37	1·30	1·32	96
Cyanide, 1 mM.	α-ketoglutarate	1·50	3·94	1·00	1·55	77
Cyanide, 1 mM.	succinate	1·63	2·34	1·10	1·78	4
Cyanide, 1 mM.	fumarate	1·59	2·30	1·38	1·46	89
Cyanide, 1 mM.	malate	1·51	2·02	1·10	1·28	65
Malonate, 20 mM.	pyruvate	1·50	3·18	1·22	2·88	1
Malonate, 20 mM.	citrate	2·00	2·54	1·45	1·68	57
Malonate, 20 mM.	*iso*-citrate	1·50	1·95	1·24	1·45	53
Malonate, 20 mM.	α-ketoglutarate	1·45	3·68	1·15	1·50	85
Malonate, 20 mM.	succinate	1·28	1·83	0·91	1·32	25
Malonate, 20 mM.	fumarate	2·38	3·35	2·00	2·27	72
Malonate, 20 mM.	malate	1·38	1·90	1·44	1·63	64

With regard to the organic acids, cyanide proved to be the most effective inhibitor, having marked action in all cases except succinate. Only with *iso*-citrate did azide produce much inhibition and in a number of cases stimulated the substrate effect. Malonate gave effects which were similar to those produced by cyanide.

It was difficult to assess the effect of these compounds upon organisms of strain 3. With a few substrates, results were similar to those obtained with the main strain. In a number of cases inhibitions were obtained but all the animals were always damaged and therefore the results are not reported; this was particularly the case with malonate. Where only 25 p.c. or less of the animals were damaged, attention to this has been drawn in the appropriate column (Table 8).

In this Table, only results which differed from those in Table 7 have been presented. The main purpose of Table 8 is to show how atypically the organisms could at times behave; the main strain was quite consistent in its reactions.

TABLE 8.

Effect of inhibitors on strain 3.

Both the polarograph and Warburg manometers were used.

Inhibitor	Substrate (10 mM.)	*E*	*S*	*I*	*SI*
Azide, 0·1 mM.	pyruvate	1·91	2·44	2·76	2·90
Azide, 0·1 mM.	*cis*-aconitate	2·64	2·40	2·52	2·80
Azide, 0·1 mM.	*iso*-citrate	1·27	1·93	1·14	1·31*
Azide, 0·1 mM.	α-ketoglutarate	1·91	2·48	2·76	2·92
Azide, 0·1 mM.	succinate	3·14	3·05	3·82	2·91*
Azide, 0·1 mM.	fumarate	3·14	2·62	3·82	3·02*
Azide, 0·1 mM.	malate	1·97	2·11	2·52	1·96*
Azide, 0·1 mM.	oxalacetate	3·14	3·31	3·82	2·96*
Azide, 0·1 mM.	acetate	3·14	3·40	3·82	4·17
Azide, 0·1 mM.	propionate	1·42	2·47	1·12	2·60
Azide, 0·1 mM.	butyrate	2·64	2·98	2·52	3·00
Azide, 0·1 mM.	glucose	2·06	2·82	2·61	3·54
Azide, 0·1 mM.	lactate	2·64	3·44	2·52	3·60
Azide, 0·1 mM.	glycine	2·64	2·18	2·52	2·20
Cyanide, 1 mM.	pyruvate	2·53	2·53	3·47	3·35*
Cyanide, 1 mM.	*iso*-citrate	1·94	2·11	1·94	2·06
Cyanide, 1 mM.	succinate	3·28	4·13	4·02	4·26
Cyanide, 1 mM.	oxalacetate	3·28	3·96	4·02	4·62
Cyanide, 1 mM.	acetate	2·53	3·55	3·47	4·49
Cyanide, 1 mM.	glucose	1·94	2·69	1·94	2·16
Malonate, 10 mM.	pyruvate	2·06	2·35	2·14	2·22
Malonate, 10 mM.	acetate	3·31	3·34	3·30	3·32
Trans-aconitate, 10 mM.	succinate	2·36	2·76	2·52	3·52
Trans-aconitate, 10 mM.	acetate	1·88	4·28	2·04	4·60
Trans-aconitate, 10 mM.	propionate	2·36	3·44	2·52	3·36
Trans-aconitate, 10 mM.	butyrate	1·92	3·57	2·12	3·66
Trans-aconitate, 10 mM.	glucose	2·14	2·50	2·30	2·62
Trans-aconitate, 10 mM.	lactate	2·08	3·52	2·27	3·68
Trans-aconitate, 10 mM.	glycine	2·64	2·34	3·38	2·48

* animals damaged.

The results with strain 3 in the presence of both cyanide and azide were quite inconsistent. Sometimes cyanide increased the activating effect of azide ($E = 2{\cdot}42$, azide $= 2{\cdot}97$, cyanide $= 2{\cdot}00$, both $= 2{\cdot}97$) whereas on other occasions there was a diminution ($E = 2{\cdot}56$, azide $= 2{\cdot}88$, cyanide $= 2{\cdot}16$, both $= 2{\cdot}32$). Methylene blue was only partially able to offset the cyanide inhibition, e.g. $E = 1{\cdot}76$, methylene blue$= 2{\cdot}74$, $I = 1{\cdot}30$, both $= 1{\cdot}70$.

DISCUSSION.

Form and motility.

Shaw and Geppert (1937) studied the effect of many toxic agents on *P. caudatum* and blistering was frequently observed. With our experiments, urethane was the most effective compound in this regard. Azide caused the

animals to swell, and this has been previously noted by Boell (1946) who found that, in *P. calkinsi*, the activity of the contractile vacuoles was reduced and there was difficulty in maintaining normal water balance. With malonate, our animals appeared quite normal; if malonate enters the cell and is to be regarded as an effective inhibitor of the citric acid cycle, then it must be concluded that the operation of the cycle is not necessary for the normal functioning of the organism.

Trans-aconitate was also used in the hope that it might act as a competitive inhibitor of the cycle at the *cis*-aconitate stage; as with malonate, *trans*-aconitate had no effect on the animal's well-being. The results with cyanide and the other compounds define the range of concentrations which can be used in metabolic experiments without affecting the viability of the animals.

The protective effect of amino-acids against azide is discussed at a later stage.

Growth.

In the absence of an added food-supply, cyanide and azide markedly affected the growth of the wild culture, bacteria and moulds being suppressed in addition to the protozoa. Where the animal's food supply was maintained the effect of azide was still considerable but cyanide was much less effective. Arsenite and selenite were also very powerful growth inhibitors.

Endogenous respiration.

The compounds produced large inhibitory effects when there was also interference with the physiological working of the organism, e.g. 0·1 mM. arsenite, 10 mM. cyanide, 10 mM. azide and 1·0 mM. azide each depressed O_2 uptake by at least 60 p.c. In the range where there was no obvious physiological interference, azide, cyanide, fluoride, malonate and *trans*-aconitate gave only moderate inhibition. Van Grembergen and Reynaerts-de Pont (1952) found no inhibition with malonate (10 mM.) but marked inhibitions with cyanide (1 mM. gave 85 p.c.) and azide (10 mM. gave 75 p.c.). No description of the organisms at the end of the experiments is given. Boell (1945) obtained a 50 p.c. inhibition of O_2 uptake wth *P. calkinsi* in the presence of 10 mM. azide and stated that after about an hour the azide began to stimulate; again no indication of the condition of the animals was given. In 1946, Boell stated that the stimulating effect is a function of pH, viz. at pH 6·02, inhibition was 70 p.c., at pH 6·24, 30 p.c., while at pH 6·59 stimulation was 138 p.c. Our experiments with *P. caudatum* did not show this pH dependence; where stimulation occurred (strain 3), change in pH did not produce inhibition. With the flagellate, *Astasia klebsii*, von Dach (1942) had previously noted a substrate and pH effect with azide, i.e. in the presence of acetate, 2 mM. azide had no effect at pH 7·9 but inhibited respiration by 95 p.c. at pH 5·8; without acetate, azide had no effect at either pH. The effects with azide are very difficult to interpret

since this agent not only affects electron carriage through cytochrome oxidase but can also affect phosphorylation (Hotchkiss, 1944).

The organisms cultured in cyanide showed more cyanide-stable respiration than normal animals and this can be explained either as a selection of those more resistant to cyanide or as a change in the metabolic equipment, following greater use of cyanide-stable mechanisms. Further, starved normal animals were more sensitive to cyanide than were normal organisms. Following the discussion of cyanide-stable respiration given by Commoner (1940) the reverse would be expected since this author maintains that the "basic" endogenous respiration is more cyanide-stable than the "substrate" respiration which occurs in well-fed animals or animals suspended in substrate solutions, e.g. glucose. Because our starved organisms were in good physiological condition we suggest that their greater cyanide sensitivity was due to an exhaustion of substrates for NH_3 production, a process (*vide infra*) which these organisms can carry out uninhibited by cyanide.

Substrate respiration.

Azide and selenite markedly inhibited glucose oxidation whereas cyanide, fluoride and malonate gave only moderate inhibition. The activating effect of arsenite can be interpreted here, as in other tissues, to be an increased glucose metabolism due to the interference in keto-acid breakdown (Dresel, 1927; Krebs, 1933).

With fatty acids as substrates, most of the inhibitors proved very powerful in their action, but with the remainder, stimulation occurred. Particularly interesting is the effect of acetate in overcoming the malonate inhibition of the endogenous O_2 uptake. The stimulation in the presence of propionate and either cyanide or azide shows that the metabolism of this fatty acid is quite different from that of acetate.

The effects with amino-acids show that these compounds are very important in the economy of these organisms since they provide pathways of metabolism alternative to those blocked by cyanide and azide. As previously found (Geddes and Humphrey, 1951) there is no compensating increase in glycolysis in the presence of cyanide or azide.

Van Grembergen and Reynaerts-de Pont (1952) showed that malonate was ineffective in inhibiting increases in O_2 consumption brought about by pyruvate and succinate. Our results confirm this but show that for the other citric acid cycle substrates used, marked inhibitions were obtained. In other invertebrates malonate has been shown to be effective in blocking succinate oxidation in only two cases; firstly, in oyster muscle (Humphrey, 1947) and secondly, in nematode parasites (Massey and Rogers, 1950). In *P. caudatum* the inhibiting effect is most marked in homogenates (Humphrey and Humphrey, 1948). Cyanide reacted similarly to malonate with the intact organisms except that succin-

ate was the only case where inhibition was not obtained; again, the work with homogenates had shown that cyanide could inhibit the succinate respiration. The results with azide indicate that this agent is unable to depress the increases in O_2 uptake observed in the presence of substrates.

The results with strain 3 show that azide, cyanide, malonate and *trans*-aconitate can stimulate the metabolism of substances of all types.

Conclusions.

It can be stated that *P. caudatum* can carry out the following types of metabolic reactions: (a) glycolysis; (b) amino-acid metabolism; (c) citric acid cycle; (d) fatty and organic acid reactions. The evidence for this is as follows:

(a) the production of lactic acid from carbohydrates and their derivatives (Geddes and Humphrey, 1951).

(b) The endogenous production of NH_3 and its stimulation by added amino-acids; the ability of amino-acids to offset inhibitions of O_2 consumption.

(c) The stimulation of O_2 uptake by citric acid cycle intermediates; the action of malonate in inhibiting some of the endogenous O_2 uptake and nearly all of the increased O_2 uptake in the presence of substrates.

(d) These reactions are postulated without a firm basis but the activation of fatty acid respiration brought about by some inhibitors and the effects of azide on some of the organic acids mean that these types of metabolic reactions which are known to occur in other organisms (Ajl, 1951) must not be neglected.

It is suggested that, with all these different pathways capable of operating, the addition of a substrate for one of them brings this pathway to the fore, e.g. when a citric acid cycle intermediate is added the operation of the cycle is stimulated and in most cases, inhibitors of the cycle (e.g. malonate) show an action characteristic of their effect on other tissues. In the intact protozoan without added substrate, malonate has no effect on the animal's well-being but one-third of its respiration is blocked. With the addition of acetate, which is the compound by means of which carbon atoms are normally brought into the cycle, suppression of the cycle with malonate would reduce the supply of oxalacetate and thus an increased O_2 uptake might be needed to produce this keto-acid (e.g. from aspartic acid) for the condensation reaction. The reason why malonate does not increase the pyruvate respiration, may be because pyruvate can react with CO_2 to form oxalacetate.

The effects of cyanide are taken to be on cytochrome oxidase and in concentrations where this compound did not damage the organisms about one-third of the O_2 uptake is prevented and in general, complete inhibition of O_2 uptake

caused by most substrates was obtained. Succinate, propionate, amino-acids and to a slight extent, glucose, are exceptions. Of these, amino-acids are the most important because their increased metabolism is called upon to offset the inhibitions in the other pathways normally used. It is noteworthy in this regard that cyanide-grown animals have a respiration more cyanide-stable than is normal. The fact that starved animals show a bigger inhibition may mean that their amino-acid reserves have been used up more than their reserves of those substrates which are metabolized through cyanide-sensitive systems.

The effects with azide are much more complicated since not only can this compound affect enzymic reactions (electron transfer, phosphorylation, etc.) but also in *P. caudatum* it prevents the normal functioning of the contractile vacuoles and depresses the growth rate. It is possible but not certain that the interference with the physiological systems is brought about by the changes in biochemical activity listed above. Azide stimulates the metabolism of some citric acid cycle intermediates, particularly the highly reactive α-keto-acids, pyruvic and ketoglutaric.

In short then, *P. caudatum* can carry out the active metabolism of many substrates so that one particular metabolic pathway is not emphasized at the expense of others as it is in higher animals. This means that there is relatively less interference by inhibitors unless the metabolism is directed into one pathway by a preponderance of added substrate. Where no substrate is added there is no extensive inhibition without structural damage.

SUMMARY.

By studying the effect of a number of compounds on the motility, morphology and O_2 consumption of *P. caudatum* with and without the addition of substrates, it has been shown that this animal can carry out reactions involving the citric acid cycle, the production of ammonia from amino-acids and possibly other types of metabolic changes using organic and fatty acids.

Because of the diversity of metabolic pathways which can be simultaneously used, respiratory inhibitors usually have only a moderate effect; when substrates are added and metabolism is channelled into one pathway, appropriate inhibitors become more effective.

Certain strains of *P. caudatum* were found to be aberrant in their reactions and it is particularly important to observe the condition of the animals after the experimental period lest misleading observations are made.

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INSULIN TOLERANCE AND HYPOGLYCAEMIC CONVULSIONS IN SHEEP

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Intravenous injection of insulin into ruminants leads to reduction of the concentration of blood sugar without precipitating typical hypoglycaemic symptoms (Bodansky, 1923; Strand, Anderson and Allcroft, 1934; Krzywanek and Hofmann, 1937; Hitchcock and Phillipson, 1946). Cutler (1934), who administered large doses of insulin to goats by intravenous injection, noted only signs of lassitude after they had been rendered hypoglycaemic for a period of 8–9 hours, and interpreted this behaviour as further evidence of a peculiarity in the carbohydrate metabolism of ruminants. Reid (1951) presented further evidence to show that the response of sheep to intravenous injections of insulin differs from that of non-ruminants; he noted that doses of insulin sufficient to produce hypoglycaemic convulsions in non-ruminants often failed to elicit these symptoms in adult sheep.

Relatively small doses of insulin given by subcutaneous injection to diabetic sheep were observed to produce hypoglycaemic coma (Jarrett, 1946), and it is probable that intravenous insulin is rapidly destroyed and its action not merely terminated by the counteraction of hypoglycaemia (Drury and Greeley, 1939).

As the carbohydrate metabolism of young lambs during the first few weeks of life is, in some respects at least, very similar to that of non-ruminants (McCandless and Dye, 1950; Jarrett and Potter, 1952) the young lamb might be expected to behave like non-ruminant animals in its response to injected insulin.

The following study was undertaken to throw further light on these phenomena.

METHODS.

A group of adult merino ewes (age 3–5 years) and a group of male and female lambs (age 1 day to 3 months) were used in these experiments. The adrenal glands of three lambs, aged 4 weeks, and of two ewes, aged 4 years, were denervated by severing the splanchnic nerve and any detectable branches of it on each side. The response of these animals to injected insulin was observed during the post-operative period. Six rabbits were treated either with intravenous or subcutaneous injections of various amounts of insulin to allow a comparison to be made between their reactions and those of the sheep. Regular insulin (Burroughs Wellcome) was administered in all cases.

The concentration of total reducing sugar in the blood drawn from the jugular vein was determined by the method of Shaffer and Somogyi (1933) using the zinc hydroxide method for the precipitation of blood proteins (Somogyi, 1930). Some of the blood samples, taken during hypoglycaemia at the onset of convulsions, were subjected to chromatographic analysis for the determination of the presence of sugar (Jarrett and Potter, 1952).

RESULTS.

Effect of intravenous insulin on adult sheep. Large amounts of insulin administered by intravenous injection into five normal sheep that had previously been fasted 1–4 days failed to bring about convulsions although the blood sugar level was reduced to below 10 mg./100 ml. for several hours. Doses of 4 units of insulin/kg. were sufficient to lower the blood sugar to this level, and by increasing the dose and giving two or three injections at intervals the concentration of sugar in the blood could be maintained below 10 mg./100 ml. for periods of 15–18 hours. One animal (No. 35) which was given three injections of 10 units/kg. at four-hourly intervals developed no obvious hypoglycaemic symptoms, although the concentration of sugar in its blood, which was determined at hourly intervals, was never above 10 mg./100 ml. during a period of 15 hours (see Fig. 1).

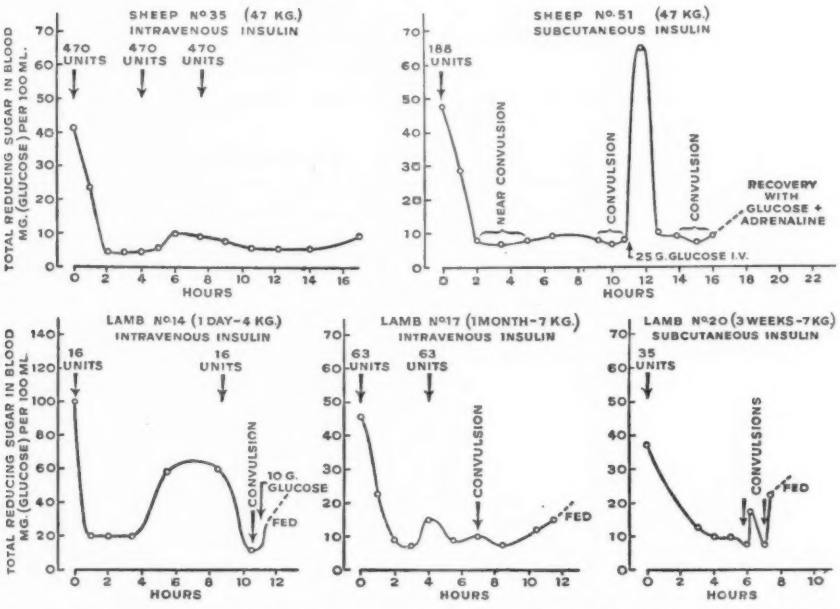

Fig. 1. Curves showing the concentration of sugar in the blood of sheep and lambs after the injection of insulin.

Effect of intravenous insulin on splanchnicotomized sheep. Two adult sheep in which both adrenals were denervated by cutting the splanchnic nerves were much more sensitive to insulin administered intravenously. Convulsions usually occurred after the concentration of sugar in the blood had been reduced to 10 mg./100 ml. and maintained at this value for a period of 3–6 hours. On most occasions a single injection of 4 units/kg. was sufficient to precipitate convulsions.

Effect of intravenous insulin on lambs. Six unsuckled lambs a few hours after birth convulsed within eight hours after insulin (4 units/kg.). The initial blood sugar level in these lambs was 80–100 mg./100 ml. and convulsions occurred when the level was about 10 mg./100 ml.

Four lambs 1–4 days old that had been allowed to suckle normally during this period and then fasted for 4–6 hours, convulsed if sufficient insulin was given; a second injection of 4 units/kg. repeated about 8 hours after the first injection, however, was usually necessary to bring about convulsions.

Twelve older lambs (1–2 months) fasted for 14 hours prior to the injection required still more insulin to produce convulsions—injections of 8 units/kg. repeated once or twice at four-hourly intervals were necessary in most cases. This apparent resistance to insulin became even more pronounced as the animals grew older, until the intravenous injection of insulin elicited no convulsive signs.

Effect of intravenous insulin on splanchnicotomized lambs. Three lambs (4 weeks old) in which the splanchnic nerves had been sectioned were, like the mature sheep treated in this way, more sensitive to insulin than a normal lamb of comparable age. These convulsed much sooner after they were injected, and usually much less insulin was required to precipitate convulsions.

Effect of subcutaneous insulin on adult sheep. Of seventeen adult sheep which were treated with a subcutaneous injection of 3–5 units/kg., fourteen convulsed. The remaining three became comatose and were then given glucose by intravenous injection. The convulsions usually occurred about 8–10 hours after the insulin had lowered the blood sugar level to below 10 mg./100 ml. Eight of the fourteen sheep that convulsed failed to recover although 25 gm. of glucose were administered by intravenous injection at intervals of a few hours; some of these, however, had been convulsing for a period of 1–5 hours before they were treated with glucose. Apparently an irreversible effect had been produced in these animals, as the blood sugar level was usually normal at the time of death. One animal (No. 36) partially recovered but continued in a state of stupor during which it bumped blindly into objects, and ingested water as though it were eating. This incoordination improved slightly after ten days, but the animal never again behaved normally.

If glucose was given immediately convulsions began, progress was much more favourable. Six of the animals recovered from convulsions even though the manner of their recovery varied considerably. Some of these improved

immediately, whereas others remained comatose for 12–20 hours, during which period their blood sugar was often normal. Three animals which received glucose and adrenaline injections seemed to recover more rapidly than those which received glucose alone.

Effect of subcutaneous insulin on lambs. Ten lambs received a subcutaneous injection of 3–5 units/kg., and eight of these convulsed; four within 2–3 hours of the injection, two after 4–6 hours and two after 7–8 hours. In every case the level of glucose in the blood at the time of convulsion was 5–7 mg./100 ml. The lambs convulsed severely for several minutes, recovered for a short period, and then convulsed again. This sequence of events occurred at brief intervals, the intervening recovery period being much more prolonged if glucose was administered intravenously. If the lamb was allowed to suckle the ewe, convulsions ceased entirely, but spontaneous recovery often occurred if the convulsions had not been too severe or prolonged.

Effect of insulin on rabbits. At least 10 units of insulin/kg. by intravenous injection or 3–5 units/kg. by subcutaneous injection were necessary to produce convulsions in rabbits (2–3 kg.) which had been previously fasted for 24 hours. A period of hypoglycaemia of about 3–4 hours was terminated by the convulsive seizures characteristic of non-ruminants. The blood sugar level just prior to a convulsion was about 5 mg./100 ml.

Details of the results obtained with representative ruminants are shown in Fig. 1 and a summary of the results presented in Table 1.

TABLE 1.

Animals	Route of insulin injection and amount administered per kg. body wt.	Duration of hypoglycaemia (hours)	Minimum blood sugar level mg. (glucose)/100 ml.	Per cent. of animals convulsing
5 adults (normal)	intravenous 20–30 units (divided doses)	12–18	5	nil
2 adults (splanchnic section)	intravenous 4 units	3–6	10	100
6 lambs (1 day old)	intravenous 4 units	6–8	10	100
4 lambs (2–4 days)	intravenous 8 units (divided doses)	8–12	10	100
12 lambs (1–2 months)	intravenous 16–24 units (divided doses)	6–12	10	83
3 lambs (splanchnic section) (1–2 months)	intravenous 6–8 units	2–3	10	100
17 adults (normal)	subcutaneous 3–5 units	8–10	7	82
10 lambs (1–2 months)	subcutaneous 3–5 units	2–8	5	80

DISCUSSION.

The results reported here confirm previous findings that the adult sheep, in common with other ruminants, is relatively insensitive to insulin administered by intravenous injection (Strand, Anderson and Allcroft, 1934; Hitchcock and Phillipson, 1946 and Reid, 1951). Although large doses by this route are required to reduce the concentration of sugar in the blood to 10 mg./100 ml. or less and maintain it at this value for several hours, adult sheep treated in this way seldom exhibit any of the usual symptoms of extreme hypoglycaemia.

The very young lamb, however, is sensitive to intravenous insulin and this sensitivity diminishes as the animal becomes older. Only one or two injections of 4 units of insulin/kg. were necessary to induce convulsions in very young lambs 1–2 days old, whereas three injections of 8 units/kg. were usually required to induce convulsions in older lambs (4–8 weeks). Mature animals seldom convulse even after injections of greater amounts of insulin.

The highly variable carbohydrate reserve of the young lamb once it has suckled is a factor to be considered, but the increasing tolerance to "convulsive doses" of insulin as the lamb becomes older seems undoubtedly to be distinct from any effect due to alimentary hypoglycaemia. Greater amounts of insulin are required to produce hypoglycaemic distress in older lambs even when they have begun to consume fodder and are thus less dependent on milk as a source of carbohydrate. At this later stage of development, the concentration of sugar in the blood is about the same as that of the adult sheep, and the lamb has already acquired some of the features of carbohydrate metabolism that are characteristic of the adult ruminant. The data obtained from these experiments with young lambs lend further support to the suggestion that the young lamb resembles a non-ruminant in some of its metabolic activities (McCandless and Dye, 1950; Jarrett and Potter, 1952).

The effect of splanchnicotomy presents some interesting features. After both splanchnic nerves had been severed the adult sheep was much more sensitive to insulin. Doses which normally produce no hypoglycaemic symptoms were then sufficient to precipitate convulsions. In the lamb splanchnicotomy produced a response similar to that of the adult; convulsions occurred after much less insulin and after a much shorter period of hypoglycaemia. This response to denervation of the adrenals agrees with that obtained by Dworkin (1931) and Brouha, Cannon and Dill (1939) who showed that sympathectomized cats are more sensitive to insulin than normal cats. Strand, Anderson and Allcroft (1934) and Reid (1948) reported a similar effect in a bilaterally adrenalectomized sheep.

The difference between a normal adult sheep which can tolerate a longer period of hypoglycaemia without convulsing, and an animal in which both adrenals have been denervated and so rendered more prone to convulsions, suggests that the resistance of sheep to intravenous insulin is partly related to

the state of the adrenal gland. Cannon, McIver and Bliss (1924) postulated that the body is protected from the effects of dangerous hypoglycaemia by a primary stage consisting of sympathetic stimulation with concurrent adrenal secretion and mobilization of sugar from the liver; and by a secondary stage, which operates only if the primary stage is inadequate, and this serves to intensify the first stage by convulsive seizures. In the adult sheep receiving intravenous insulin either the action of the insulin is terminated before a convulsive state is reached or sympathetic discharges stimulate adrenal secretion as the threshold is approached and convulsions do not occur.

The responses to insulin administered by subcutaneous injection into both adult sheep and lambs differed considerably from those observed after intravenous insulin. One outstanding feature was the long period of hypoglycaemia which persisted after a relatively small subcutaneous injection of insulin followed by convulsions in the adult sheep. Once convulsions had begun, the adult often lapsed into an apparently irreversible state which was not relieved by intravenous glucose.

Dotti (1934) has claimed that convulsions occur only when the blood is ostensibly free of glucose. The results reported above, however, indicate that an anomaly exists between the effects of insulin when administered to adult sheep by intravenous and by subcutaneous injection. After intravenous insulin the blood contained too little sugar to be estimated by the method used, and yet no convulsions ensued, whereas after subcutaneous insulin at least 7–10 mg./100 ml. were present for most of the hypoglycaemic phase when convulsions occurred. The presence of glucose in the blood of these animals was proven by our colleague J. Frahn who, by the use of quantitative chromatographic methods, found amounts approximately equal to those indicated by the direct analytical procedures.

Reid (1951) has claimed that in the sheep the blood sugar could not readily be depressed to a sufficiently low level to induce hypoglycaemic symptoms. However, from the results reported here it is apparent that merely reducing it to a very low level could not be in itself directly responsible for the onset of convulsions (cf. Fig. 1, sheep No. 35 and 51). This is further substantiated by the experiments in which insulin was administered intravenously to splanchnicotomized sheep.

Peters and Van Slyke (1946) have discussed the possibility that hypoglycaemic symptoms may be the result of a low concentration of glucose in the tissues which is not completely reflected by the level of the blood sugar. The seizures which continue in a convulsing sheep after the blood sugar has been elevated by intravenous injection of glucose, might be explained if this were true, but it seems evident that other factors are operating. This behaviour which usually precedes the apparently irreversible state is interesting in view of the fact that both lambs and rabbits after a "convulsing dose" of insulin

administered subcutaneously have a much shorter latent period before convulsions begin, and usually recover spontaneously unless convulsions are prolonged.

On three occasions when the sheep was apparently approaching an irreversible state, a better clinical response resulted after simultaneous injections of glucose and adrenaline. Gellhorn (1943) has discussed the influence of adrenaline on hypoglycaemic convulsions and has observed that convulsions could be abolished in rabbits by amounts of adrenaline too small to raise the blood sugar. If this is so, the effect of adrenaline might be associated in part, at least, with changes in cerebral circulation following pressor reflexes elicited from the carotid sinus (Gellhorn, Yesinick and Darrow, 1940).

The clinical picture presented by sheep in this irreversible state was very similar to that reported for other animals. Sheep No. 36 which remained in a state of incoordination for some weeks showed most of the reactions described by Sherrill and MacKay (1939) for non-ruminants. There can be little doubt that its carbohydrate metabolism and the circulation of glucose in its tissues could not have continued to be influenced for such a period.

The behaviour of sheep to either intravenous or subcutaneous insulin before and after splanchnicotomy, and the difference in tolerance to extreme hypoglycaemia between the young and the adult present a number of interesting problems which are not yet solved. In view of the prolonged hypoglycaemic phase which follows a subcutaneous injection of insulin into adult sheep it seems probable that the main effect is related to the intermediary metabolism of carbohydrate in the tissues and especially in the central nervous system of the ruminant.

SUMMARY.

Adult sheep do not convulse after the injection of massive doses of insulin into the systemic circulation, even though the concentration of sugar in the blood is reduced to very low levels for long periods.

Denervation of the adrenal glands renders the adult sheep sensitive to the intravenous injection of insulin, and convulsions ensue after small doses.

Relatively small doses of insulin injected subcutaneously into adult sheep are followed by long periods of hypoglycaemia terminating in severe and prolonged convulsions.

Young lambs are much more sensitive to intravenous insulin than adult sheep; tolerance increases as the lambs become older.

The young lamb behaves like a non-ruminant in its response to both intravenous and subcutaneous insulin.

Once convulsions begin in adult sheep after subcutaneous insulin, an apparently irreversible state is often reached which is usually refractory to intravenous glucose therapy in spite of a return to normal blood sugar level.

Acknowledgments. Portion of this work was begun using young calves while one of us (I. G. J.) was on a visit from C.S.I.R.O. to the Physiological Laboratory, Cambridge, during 1950–51. Our thanks are due to Dr. R. S. Comline and Mr. D. A. Tichen for their early help and interest in the splanchnicotomy. Acknowledgment is also made to Mr. H. R. Marston, F.R.S., Chief of the Division, for his active encouragement.

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DEAMINATION OF ASPARTIC ACID BY *PROTEUS* X-19

II. ASPARTASE ACTIVITY OF TOLUENE-TREATED CELLS

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One effect of toluene on bacteria like *E. coli* and *Proteus* is to abolish the ability of materials such as adenosine and glucose to stimulate the anaerobic deamination of aspartic acid (Gale, 1938; Trudinger, 1951). The activities of toluene-treated cells recorded by these workers were very low compared with those of whole cells, and were attributed by Gale to a separate enzyme which contributed only in part to the deamination by whole cells. However, it was observed by the author that the activity of toluene-treated cells can be increased greatly under various conditions so that an elucidation of its relationship to the activity of whole cells becomes of major importance.

A detailed investigation of the activity of toluene-treated cells is reported in this paper.

Materials and Methods.

Most of these have been described in a previous paper (Trudinger, 1951).

To test the generality of the findings reported below, the major experiments were performed not only on *Proteus* but also on *E. coli* and *A. aerogenes*, both strains obtained from the Bacteriology Department, University of Adelaide. Unless otherwise stated, the results reported have been obtained with *Proteus*.

The growth medium was similar to that described in 1951 except for the addition of 0·5 to 1 p.c. yeast extract (Difco), which increased the yield of organism (activities, however, were virtually unchanged). Malate (and fumarate) were estimated by decarboxylation with *Lb. arabinosus* (and fumarase) as described by Nossal (1951).

Unless otherwise stated, all incubations were carried out for 1 hour at 38° C. with shaking. $Q_{NH_3} = \mu l$ NH_3 liberated per hour by 1 mg. dry wt. of cells. All results have been corrected for their appropriate blanks. Toluene-treated cells are referred to henceforth as T.T. cells.

RESULTS.

Toluene treatment.

Removal of toluene from the suspension after treatment does not reverse its action nor does its presence during deamination have any adverse effect on the reaction. Concentrations of toluene from 0·5 p.c. upwards are equally effective in producing the metal requirement to be described below; a saturated solution, however, is sufficient to abolish completely any stimulation by glucose, etc. The standard procedure adopted for toluene treatment was as

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follows. The cells were suspended at the required strength in buffer (or other medium) and shaken vigorously with 1 p.c. toluene on a mechanical shaker for five minutes at room temperature. This produced an even suspension of toluene which remained stable for about 5 minutes, during which time the cells were pipetted into the reaction mixture.

Since toluene affects a number of colorimetric and enzymatic estimations, the cells were freed of toluene in those experiments where such methods were employed. In these cases, thick suspensions were shaken with 10–20 p.c. toluene, centrifuged and the cells washed once in buffer before use. (It was found necessary to dry the internal wall of the centrifuge tube with tissue paper between each washing to remove toluene which adhered there.)

Considerable amounts of fatty material were extracted during this procedure and collected in the toluene layer on centrifugation. Otherwise the cells appeared to be little altered, retaining their general shape and staining properties.

Effect of metal inhibitors on aspartate activity.

A number of substances which are known to form complexes with metallic ions (see for example Albert and Gledhill, 1947) have marked inhibitory actions on the deamination of aspartic acid by whole cells (Table 1). In general, the inhibitions of basic and stimulated activities are of the same order, but notable exceptions are 8-hydroxyquinoline and cupferron, both of which almost abolish adenosine stimulation at concentrations which allow significant basic activity and stimulation by glucose.

TABLE 1.

Effect of metal-combining agents on the anaerobic deamination of aspartic acid.

0·0025 M L-aspartate; 0·06 M phosphate pH 7·0; 0·24 to 0·35 mg. cells; anaerobic. Additions: glucose or adenosine, 10^{-3} M.

Expt.	Inhibitor	Concentration	No. additions (a)	Q_{NH_3} Glucose (b)	Adenosine (c)	(b)−(a)	(c)−(a)
1.	None		133	288	293	157	162
	8-hydroxyquinoline	2×10^{-3} M	83	143	87	60	4
	8-hydroxyquinoline	10^{-3} M	100	173	113	73	13
2.	None		89	226	237	137	148
	Picrolonic Acid	10^{-3} M	27	114	111	87	84
3.	None		81	209	222	128	141
	α-α′-dipyridyl	10^{-4} M	20	64	58	44	38
4.	None		103	265	272	162	169
	Cupferron	10^{-3} M	34	135	48	101	15
5.	None		120	296	301	176	181
	Thioglycollic Acid	10^{-3} M	101	246	255	145	154
6.	None		116	240	240	124	124
	Sodium diethyldithiocarbamate	10^{-3} M	78	194	194	116	116
7.	None		127	257	278	130	151
	Fluoride	2×10^{-2} M	62	117	123	55	61

It seemed likely, therefore, that some metal is involved in the deamination reaction (and probably also in the stimulatory mechanisms).

Effect of divalent metals on aspartase activity.

The aspartase activity of T.T. cells is under certain conditions strongly stimulated by the addition of Mg^{++}, Mn^{++} or Co^{++} (Table 2); these metals have little effect on freshly prepared whole cells (Expt. 1A). This stimulation, however, is only manifest when relatively low concentrations of aspartic acid are employed (Table 3). Thus, at a substrate concentration of 0·0025 M, *Proteus* and *A. aerogenes* exhibit marked metal dependence and *E. coli* moderate dependence, while at aspartate levels four to eight times as high, no stimulation (in some cases even slight depression) of activity is observed. This effect is partially due to the increased salt concentration introduced with aspartate (see below). In Experiment 2 of Table 3, the effect of an incinerated preparation of neutralized aspartate was investigated. The incinerated preparation (designated IP in the Table) was made by evaporating to dryness in a crucible an acidified (HCl) aliquot of the sample used in other experiments, and igniting until all carbon had disappeared. The crucible was then extracted with the original volume of hot water. The preparation thus contained all salt material present in the original sample. This material produced significant activation of aspartase. However, whereas with *Proteus* deamination of 0·02 M L-aspartate is unaffected by Mg^{++}, the addition of IP equivalent to this concentration of aspartate did not produce more than 50 p.c. of the maximum activity found with 0·005 M aspartate. This suggested that deamination becomes less dependent on metal as the concentration of substrate increases. Table 4 shows that this is indeed so. Concentrations of KCl and $MgCl_2$, producing little or no stimulation at a substrate level of 0·0025 M, stimulate effectively deamination of 0·01 M aspartate; also, the concentration of metal required for maximum activity is decreased by the increase in aspartate.

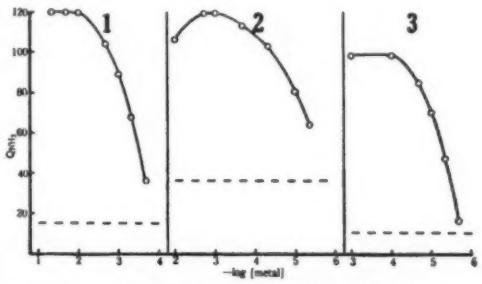

Fig. 1. Effect of metal concentration on activity of toluened cells.

0·0025 M L-aspartate; 0·066 M phosphate pH 7·0 ca. 0·5 mg. cells. Aerobic.

Curve 1. $MgCl_2$. Curve 2. $MnCl_2$. Curve 3. $CoCl_2$.

The concentrations required for optimal activity in phosphate buffer for a substrate concentration of 0·0025 M, are approximately 10^{-2} M, 10^{-2} M and 10^{-4} M for Mg^{++}, Mn^{++} and Co^{++} respectively (Fig. 1). As the concentration is decreased from the optimum, the efficiency of Mg^{++} and Co^{++} falls very

TABLE 2.

Effect of magnesium, manganese and cobalt on aspartic acid deamination.

0·0025 M L-aspartate; 0·066 M phosphate, pH 7·0.
Cells (0·56–0·66 mg.): Expt. 1A, untreated; Expts. 1B, 2 T.T.

Expt.	Metal	Final concentration	Q_{NH_3} Aerobic	Q_{NH_3} Anaerobic
1A	0	—		115
	$MgCl_2$	10^{-2} M		122
	$MnCl_2$	10^{-3} M		120
	$CoCl_2$	10^{-4} M		121
1B	0	—	9	33
	$MgCl_2$	10^{-2} M	122	120
	$MnCl_2$	10^{-3} M	102	112
	$CoCl_2$	10^{-4} M	107	100
2	—	—	5	16
	$MgCl_2$	10^{-3} M	66	86
	$MnCl_2$	10^{-4} M	93	112
	$MnCl_2$	10^{-5} M	34	62
	$CoCl_2$	10^{-5} M	25	64
	$CoCl_2$	2×10^{-6} M	5	29

TABLE 3.

Effect of substrate concentration on requirement of T.T. cells for magnesium.

0·066 M phosphate, pH 7·2; aerobic.

Expt.	Organism	Concentration of L-aspartate	Q_{NH_3} No. mg.	Q_{NH_3} 10^{-2} M $MgCl_2$
1.	*Proteus* X–19	0·0025 M	7	124
	(0·60 mg. cells)	0·005 M	27	194
		0·01 M	106	285
		0·02 M	400	403
2.	*Proteus* X–19	0·005 M	19	178
	(0·53 mg. cells)	0·005 M + IP(1)	50	
		0·005 M + IP(2)	85	
		0·005 M + IP(3)	136	
3.	*E. coli*	0·0025 M	35	66
	(1·44 mg. cells)	0·005 M	100	108
		0·01 M	168	157
4.	*A. aerogenes*	0·0025 M	18	72
	(1·2 mg. cells)	0·005 M	50	114
		0·01 M	140	171
		0·02 M	251	230

IP = incinerated aspartate preparation (see text).
(1) ≡ 0·01 M L-aspartate. (2) ≡ 0·02 M L-aspartate. (3) ≡ 0·033 M L-aspartate.

rapidly, while Mn^{++} exhibits a high activity over a relatively wide range of concentrations. The relative stimulations produced by sub-optimal amounts of metal vary somewhat with suspensions, no doubt owing to variations in metal

content of the cells. It was generally found that the lower the "basic" activity, the less marked was the effect of small concentrations of metal. At supra-optimal concentrations, Mn^{++} and Co^{++} inhibit. In borate buffer, Co^{++} is markedly less efficient than Mg^{++} or Mn^{++}; also its optimal concentration is at least five times that in phosphate buffer.

TABLE 4.

Effect of substrate concentration on magnesium and KCl requirement of T.T. cells.

0·066 M phosphate, pH 7·2; 0·59 mg. cells; aerobic.

Additions	Concentration	Q_{NH_3} 0·0025 M L-aspartate	Q_{NH_3} 0·01 M L-aspartate
None	—	8	152
$MgCl_2$	10^{-4} M	8	210
$MgCl_2$	10^{-3} M	72	258
$MgCl_2$	10^{-2} M	127	262
KCl	10^{-2} M	10	250
KCl	2×10^{-2} M	30	253
KCl	10^{-1} M	107	250

Generally, it was found that the activity in the presence of Mg^{++} was least affected by variations in conditions, and it was therefore the metal of choice for most experiments.

No increased activity is observed when two metals are added concurrently. It will be noted in Table 2 that in the absence of metal, or when metal is present in suboptimal concentration, the activity under aerobic conditions is consistently lower than anaerobically. In the presence of optimal metal concentrations, however, the activity is the same under both conditions. The reaction is equally dependent upon added metal during its initial stages.

Effect of other metals. No effect, or less than 20 p.c. of that produced by Mg^{++}, was obtained with $CaCl_2$, $FeSO_4$ (10^{-3} —10^{-2} M), $SnCl_2$, $SbCl_2$, $BaCl_2$, $ZnSO_4$ (10^{-2} M), $NiCl_2$ (10^{-5} —10^{-2} M), or $CuSO_4$ (10^{-6} M.) Inhibition of basic activity was produced by $SrCl_2$ (10^{-3} M), $CuSO_4$ (10^{-4}—10^{-3} M), CuCl (10^{-5} M), $AlCl_3$ and $CdCl_2$ (10^{-2} M).

Effect of KCl and NaCl.

KCl and NaCl stimulate the aspartase activity of T.T. cells in phosphate buffer (Table 5), the optimal concentration for 0·0025 M aspartate being *ca.* 10^{-1} M. In borate buffer, concentrations of KCl or NaCl up to 0·2 M have no effect on the deamination. Both salts stimulate equally at the same concentration, and mixtures behave as an equivalent concentration of either alone. LiCl is ineffective.

TABLE 5.

Effect of KCl and NaCl on deamination of aspartate by T.T. cells.
0·0025 M L-aspartate; 0·066 M buffer, pH 7·0; aerobic.

Expt.	Buffer	Concentration of salt	Total cation (*)	Q_{NH_3} NaCl	KCl
1.	Phosphate	0	0·15 M	19	
		5×10^{-1} M	0·65 M	84	87
		2×10^{-1} M	0·35 M	106	107
		10^{-1} M	0·25 M	107	106
		5×10^{-2} M	0·20 M	90	97
		2×10^{-2} M	0·17 M	54	55
		10^{-2} M	0·16 M	41	42

(*) of buffer + added salt.

In view of the high concentration of salt required, it became important to ascertain whether any divalent metal was present in sufficient concentration to produce these results. The following facts suggest strongly that this was not so:

1. Repeated recrystallization and passage through ion exchange resin did not decrease the activity of the salts.

2. Several samples of salts from different warehouses as well as HCl neutralized with KOH and NaOH produced the same activation patterns.

3. Flame spectrometric examination of the various salts concerned showed that contamination by Mg^{++}, Mn^{++} and Co^{++} was insignificant. The only metal contaminant in any appreciable quantity was iron. However, a wide range of concentrations of $FeCl_2$ and $FeCl_3$ failed to produce activation.

Attempts were made to determine whether the salts themselves or the Na^+ and K^+ ions were responsible for the activation. This is difficult to ascertain since many anions (sulphate, nitrate, phosphate) are strongly inhibitory at the concentrations required. Some activation was obtained with acetates, but the anion is inhibitory at 10^{-1} M. The results obtained with increases in aspartate concentration suggest that the cation may be responsible. On the other hand, significant stimulations are produced by salt additions which are very small compared with the cation concentration already present in the buffer (Table 5).

The activities produced by an optimal concentration of KCl or NaCl are usually about 20 p.c. below that effected by the divalent metals. The concurrent addition of salt and divalent metal produces the same result as the metal alone. Stimulation by KCl or NaCl is similar to that brought about by suboptimal amounts of Mg^{++}, etc., in that the anaerobic activity is always above that produced aerobically.

Action of glucose, adenosine, biotin, etc.

No stimulation of deamination by T.T. cells (with metal) has been obtained by the addition of glucose, adenosine, adenylic acid, yeast extract (Difco), glu-

tathione, lactate or biotin, or mixtures of these materials. (Yeast extract will stimulate in the absence of added metal presumably by virtue of metal contained in it.)

Stability of aspartase activity in T.T. cells.

The aspartase activity of T.T. cells suspended in distilled water is very unstable. Thirty minutes' incubation at 38° C. is sufficient to render the cells almost inactive (Table 6); at room temperature (18–22° C.), suspensions become inactive after 2–4 hours, while at 2° C. considerable reduction of activity occurs in 6–8 hours, and complete inactivation overnight. Inactivation occurs both aerobically and anaerobically. Cells incubated in distilled water prior to toluene treatment exhibit only slightly lowered activity (Expts. 2 and 3, Table 6). At 2° C., untreated cells can be kept for at least 7 days with only about 20 p.c. loss of their activity under toluene. Removal of toluene by centrifugation of the cells and resuspension in water does not decrease their instability.

TABLE 6.

Stability of aspartase activity of T.T. cells in distilled water.

Cells suspended in distilled water (*ca.* 1 mg./ml.), shaken with toluene and incubated aerobically with shaking. Aliquots taken at required intervals and tested for aspartase activity in following system.

0·0025 M L-aspartate: 0·066 M buffer (phosphate or borate), pH 7·3; 0·01 M $MgCl_2$.

Expt.	Preincubation (Minutes)	Q_{NH_3} Phosphate	Borate
1.	0	176	286
	10	37	37
	20	14	13
	30	8	9
2.	0	180	225
	30	6	6
	30*	136	167
3.†	0	120	
	30	25	
	30*	106	
4.‡	0	140	140
	20	93	75
	40	68	55

* Cells incubated in distilled water for 30 minutes *before* toluene treatment and tested for activity immediately after treatment.

† Preincubation and deamination carried out anaerobically.

‡ Frozen and thawed cells.

Cells suspended in phosphate buffer at near neutral pH are quite stable, retaining their activity for at least 2 hours at 38° C. Inactivation in borate is slower than in distilled water, but 60–70 p.c. of the initial activity is lost after one hour's incubation at 38° C. Both potassium and sodium phosphates are

effective in preventing inactivation. 0·01 M KCl or NaCl retard the inactivation, but are even less effective than borate in this respect.

Concentrations of phosphate above 0·01 M have, however, a distinct inhibitory action on the deamination reaction; 0·02 M phosphate inhibits 30 p.c. in borate. The effect of increasing phosphate buffer concentration is shown in Table 7; the activity progressively decreases to a greater degree than can be accounted for by increase in K^+ or ionic strength.

TABLE 7.

Effect of phosphate and ionic strength on activity of T.T. cells.

0·002 M L-aspartate; 0·01 M $MgCl_2$; 0·025 M phosphate, pH 7·0 ($K^+ = 0·04$ M); aerobic.

Cells (mg.): Exp. 1, 1·0; Exp. 2, 0·73; Exp. 3, 0·46.

Additions	Total PO_4^{---}	Total K^+	μ*	Q_{NH_3} Exp. 1	Exp. 2	Exp. 3
None	0·25	0·04		122	137	163
0·5 M KPO_4‡	0·525	0·84	1·10			31
0·25 M KPO_4‡	0·275	0·44	0·55	46	50	66
0·1 M KPO_4‡	0·125	0·198	0·22	72	87	
1·25 M KCl	0·25	1·29	1·25	67	78	
1·0 M KCl	0·25	1·04	1·0			71
0·5 M KCl	0·25	0·54	0·5	95	116	111
0·156 M KCl	0·25	1·96	0·156	117	137	

*$\mu = 0·5\ \Sigma C_i Z_i^2$ of added constituents only. (Potassium phosphate calculated on basis of $3K^+$ and one PO_4^{---}).

† Reaction run anaerobically.

‡ Potassium phosphate buffer pH 7·0.

On the other hand, relatively high phosphate concentrations are necessary to stabilize effectively the activity of T.T. cells. In the experiments described in Table 8, the cells were suspended in the respective sodium phosphate concentrations, toluene-treated and incubated aerobically for 30 minutes at 38° C. Sodium phosphate or distilled water, aspartic acid and $MgCl_2$ were added to give final buffer concentrations of (a) experiment 1, 0·034 M and (b) experiment 2, 0·68 M. Ammonia was estimated after 1 hour's aerobic incubation at 38° C. Control values represent the activities of the cells before preincubation. Concentrations of phosphate from 0·05 M to 0·15 M were necessary to produce reasonable stability. It will be noted that, to preserve the somewhat higher activities of aspartase found when the reaction is run in relatively low concentrations of phosphate, *higher* concentrations of phosphate are required during the preincubation period. In practice, reproducible results over a period of 8 hours were obtained when the cells were stored and the reaction carried out in phosphate buffer of 0·05 M–0·10 M. Under these conditions, cells incubated 22 hours at 38° C. lost no more than 30 p.c. of their activity.

TABLE 8.

Effect of phosphate concentration on stability of T.T. cells.

0·0026 M L-aspartate; 0·01 M $MgCl_2$; aerobic; pH 7·2.
Procedure: see text.

Expt.	Molarity of phosphate during preincubation	Molarity of phosphate during reaction	Q_{NH_3}
1.	Control	0·034	207
	0·14		190
	0·07		165
	0·007		128
	0·0007		83
	0		30
2.	Control	0·068	137
	0·14		135
	0·07		130
	0·007		92
	0·0007		50
	0		10

The experiments described above utilized buffers of approximately neutral pH. The cells are also stable at pH 6·0 in phosphate buffer, but at pH 8·0–8·5, they are almost as unstable as in distilled water (Table 9). When suspended in distilled water, inactivation occurs readily at all pH values tested (6·0, 7·0, 8·0, 9·0) although the rate of inactivation is greater at the more alkaline pH values. 100 p.c. inactivation occurs within 5 minutes at pH 8·0. No significant change in pH occurred during incubation of T.T. cells in distilled water.

TABLE 9.

Effect of pH on stability of T.T. cells in phosphate.

Cells suspended in appropriate buffer, toluene treated and incubated aerobically with shaking. At required intervals, aliquots were neutralized to pH 7·2 with Na_2HPO_4 or NaH_2PO_4 and activity determined in the usual way.

Preincubation (minutes)	Q_{NH_3} pH 6·0	pH 8·5
0	163	163
30	158	60
60	151	36

No reactivation of inactivated cells was observed on incubation of cells in phosphate with glucose, adenosine, lactate, biotin, yeast extract or extract of heat-treated *Proteus*.

The results reported were obtained with *Proteus*; the inactivation in distilled water and stabilization by phosphate were also observed with *E. coli* and *A. aerogenes*.

Effect of pH.

The pH for optimal activity in phosphate or borate is *ca.* 7·5 when Mg^{++} is used, and *ca.* 7·1 when Mn^{++} or Co^{++} is the stimulating metal (Fig. 2). This agrees with the results of Virtanen and Tarnanen (1932), who found optimal activity of aspartase from dried *B. fluorescens* to occur between pH 7·0 and 7·5. The enzyme is inactive at pH 5 and pH 9·0. In phosphate, the cells still retain activity at pH 8·0–8·5 in the presence of Mg^{++}, while they are inactive when M^{++} or Co^{++} are used.

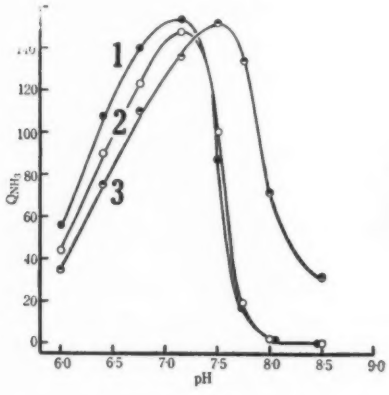

Fig. 2.

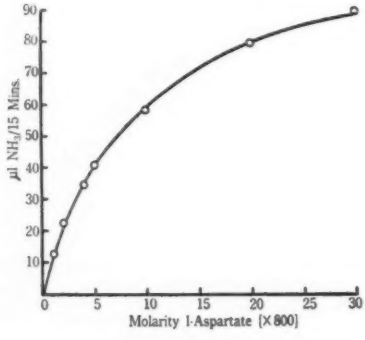

Fig. 3

Fig. 2. Effect of pH on activity of toluened cells. Buffers 0·066 M sodium phosphate; other conditions as for Fig. 1.

Curve 1. 10^{-4} M $CoCl_2$. Curve 2. 10^{-3} M $MnCl_2$. Curve 3. 10^{-2} M $MgCl_2$.

Fig. 3. Effect of substrate concentration on rate of deamination.

0·01 M $MgCl_2$; other conditions as for Fig. 1.

The marked decline in activity at pH values more alkaline than the optimal is expected, owing to the relative instability of the enzyme under these conditions. In borate buffer, the effect is even more marked, the activity falling to zero at *ca.* pH 8·0 in the presence of Mg^{++}. (The optimal pH value is again *ca.* pH 7·5 with Mg^{++}.)

The shape of the curve and the optimal pH are unaffected by the cationic constitution of the buffer; however, particularly in the presence of Mn^{++} or Co^{++}, the cells are somewhat more active in sodium buffer than in potassium buffer.

Effect of temperature. The deamination rate for an hourly period is maximal at a temperature of 37° C.–40° C. Q_{10} between 28° C. and 38° C. is approximately 1·5. At 50° C. the enzyme is inactivated within 5 minutes. Jacobsohn and Tapadinhas (1935) have reported that a decrease in temperature shifts the equilibrium towards aspartate synthesis.

Effect of substrate concentration.

A plot of initial velocity in phosphate against substrate concentration is shown in Fig. 3. Michaelis-Menten constants calculated from the equation of Lineweaver and Burk (1934) were, in three separate experiments, $8{\cdot}05 \times 10^{-3}$ M, $7{\cdot}7 \times 10^{-3}$ M and $8{\cdot}26 \times 10^{-3}$ M respectively.

In borate, activity decreases at concentrations of substrate above *ca.* 3×10^{-3} M; at $1\text{–}2 \times 10^{-2}$ M, activity ceases altogether (Table 10).

TABLE 10.

Effect of substrate concentration on activity of aspartase in borate.

0·066 M borate, pH 7·2; 0·1 M $MgCl_2$; aerobic.

	Aspartate concentration	μl NH_3/3ml./15 mins.
Exp. 1. (0·75 mg. cells)	0·00167 M	41
	0·00334 M	50
	0·00667 M	38
	0·0134 M	3
	0·02 M	0
		Q_{NH_3}
Exp. 2. (0·45 mg. cells)	0·0025 M	223
	0·0025 M + 0·01 M NaCl	223
	0·0025 M + 0·1 M NaCl	190
	0·01 M	40

Course of reaction.

Fig. 4 shows the course of deamination over 120 minutes. The rate falls rapidly as deamination proceeds. Aspartase is a reversible reaction (Quastel and Woolf, 1926; Virtanen and Tarnanen, 1932; Jacobsohn and Tapadinhas, 1935), so that the decline in activity must be partially due to the decrease in substrate concentration and approach of the equilibrium state. (Cells centrifuged and washed after as long as 22 hours in a reacting system, still possess 70–80 p.c. of their original activity; thus destruction of enzyme is not a significant factor.)

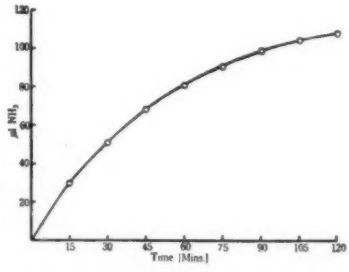

Fig. 4. Course of reaction of aspartase.

0·01 M $MgCl_2$; other conditions as for Fig. 1.

Effect of di- and tricarboxylic acids.

Fumarate strongly inhibits the rate of aspartic acid deamination (Table 11) and undoubtedly plays a major rôle in the decrease in activity with time. This inhibition (at least in the early stages of the reaction) appears to be competition with the enzyme surface rather than a mass action effect, since ammonia does not exhibit this inhibitory action *per se*, nor does it enhance the effect of

fumarate. The system exhibits the kinetics of competitive inhibition (Fig. 5). (Ammonia does produce some depression of activity when the reaction is nearing completion; the effect, however, is small compared with that produced by fumarate.) A slight depression of activity is also produced by 10^{-2} M succinate, but none by oxalacetate nor by the three tricarboxylic acids tested.

TABLE 11.

Effect of di- and tricarboxylic acids on deamination of aspartate by T.T. cells.

0·0025 M L-aspartate; 0·066 M phosphate, pH 7·0; 0·01 M $MgCl_2$.

Exp.	Conditions	Additions	Final concentration	Q_{NH_3}
1.	Aerobic	None	--	120
		Oxalacetate	10^{-2} M	130
		Succinate	10^{-2} M	88
		DL-malate	10^{-2} M	49
2.	Anaerobic	None	--	138
		Fumarate	10^{-2} M	33
		Succinate	10^{-2} M	118
		L-malate	10^{-2} M	49
		cis-Aconitate	10^{-2} M	136
		trans-Aconitate	10^{-2} M	138
		Citrate	10^{-2} M	137

Malic acid is as effective as fumarate in inhibiting the reaction. The fumarase activity of T.T. cells is very high (Q values as high as 5,000–8,000 for the first 5 minutes have been recorded with 0·01 M fumarate) so that malate and fumarate are equilibrated very rapidly. During the initial stages of the reaction, malate inhibits to a lesser degree than fumarate, the inhibition increasing as the reaction proceeds to reach that produced by fumarate (Fig. 6). Furthermore, preincubation with inhibitors before addition of substrate decreases fumarate inhibition and increases that produced by malate, the two substances exhibiting the same effect. Fumarate is thus the active material, malate acting by its conversion to fumarate.

At a substrate concentration of $2{\cdot}5 \times 10^{-3}$ M, 50 p.c. inhibition is produced by 2×10^{-3} M added fumarate, which, assuming $K_{\text{fumarase}} = 4$, represents *ca.* 5×10^{-4} M (actual) fumarate.

The effect of fumarate is the same regardless of the metal used in the reaction. Similar results were obtained with *E. coli* and *A. aerogenes*.

Optical specificity of substrate. It is generally accepted that only the laevo isomer of aspartate is attacked by aspartase in T.T. cells (Jacobsohn and Pereira, 1936; Soares, 1937). The author's results confirm this. The course of reaction and ammonia output at completion are the same whether the substrate is L- or DL-aspartate equivalent to the same concentration of the L-isomer. The D-isomer does not inhibit.

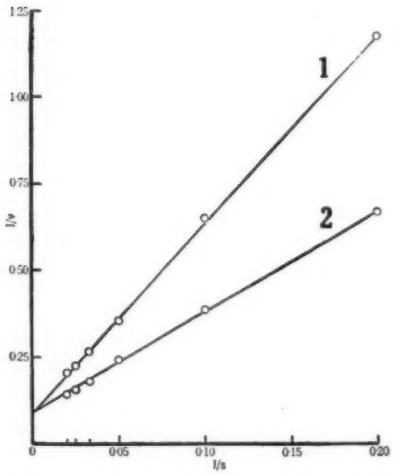

Fig. 5

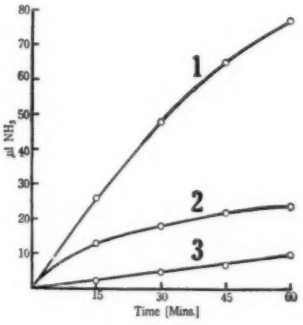

Fig. 6

Fig. 5. Competitive inhibition by fumarate.

0·1 M $MgCl_2$; 1·1 mg. cells; 0·066 M phosphate pH 7·2 Aerobic.
Curve 1. 22×10^{-3} M fumarate added. Curve 2. No fumarate.
$v = \mu$M NH_3/3 ml./15 minutes.
$s = \mu$M L-aspartate/3 ml.

Fig. 6. Course of reaction in presence of fumarate and malate. Conditions as for Fig. 3.

Curve 1. No additions. Curve 2. 10^{-2} M L-malate added. Curve 3. 10^{-2} M fumarate added.

Reaction products.

Quastel and Woolf (1926) proposed the following equation for the aspartase reaction:

$$\text{L-aspartate} \rightleftharpoons \text{fumarate} + NH_3$$

Using the more specific malic decarboxylase method for estimation of fumarate and malate, it was confirmed that ammonia and fumarate plus malate are produced in 1 : 1 proportions. However, since the fumarase activity is far in excess of aspartase, the initial product of reaction could not be determined. Attempts to produce fumarase-deficient preparations by varying growth conditions and by "ageing" of cells have as yet not proved successful. The immediate product of reaction still awaits experimental demonstration. Virtanen and Erkama (1938) claimed to have produced aspartase free of fumarase; but later Virtanen and Louhivuori (1947) state that subsequent attempts to produce fumarase-free preparations were unsuccessful.

Further metabolism of malate and fumarate is not entirely absent in T.T. cells, and gradual removal becomes evident after 3 to 4 hours under aerobic

conditions. The dicarboxylic acid almost completely disappears within 20–24 hours. This is not accompanied by a further ammonia output, and in many cases the ammonia concentration falls as well. Removal is less rapid under aerobic conditions. The fate of the dicarboxylic acid and ammonia has not as yet been investigated.

Aspartase activity in frozen and thawed cells.

Cells slow frozen at —2° C. and thawed at 38° C. exhibit many of the characteristics of T.T. cells (Table 12). The basic activity, although usually higher than in T.T. cells, is low and is stimulated by the addition of Mg^{++}, Co^{++} and Mn^{++} when low substrate concentrations are used. At 0·0025 M L-aspartate, the optimal requirements for the various metals are the same as with T.T. cells. The enzyme in frozen cells is unstable in distilled water but stable in phosphate (Expt. 4, Table 6).

TABLE 12.

Aspartase activity in frozen and thawed cells.

0·0025 M L-aspartate; 0·066 M phosphate pH 7·0; 0·4–0·5 mg. cells.

Exp.	Additions	Final concentration	Q_{NH_3} Anaerobic	Q_{NH_3} Aerobic
1.	None	— —	82	65
	$MgCl_2$	10^{-2} M	118	116
	Glucose	10^{-3} M	47	
	Glucose + $MgCl_2$	10^{-3} M + 10^{-2} M	116	
	Adenosine	10^{-3} M	51	
	Adenosine + $MgCl_2$	10^{-3} M + 10^{-2} M	116	
2.	None	— —	79	
	Glucose	10^{-3} M	39	
	Adenosine	10^{-3} M	41	
3.	None	— —	31	
	$MgCl_2$	10^{-2} M	131	
	$MnCl_2$	10^{-3} M	129	
	$CoCl_2$	10^{-4} M	125	

TABLE 13.

Variation in activity of whole and T.T. cells with substrate concentration.

0·066 M phosphate, pH 7·2; 0·0133 M $MgCl_2$; 0·5 mg. cells; aerobic; 1 hour's incubation.

L-aspartate (Molarity)	Q_{NH_3} Whole cells	Q_{NH_3} Toluened cells
0·00168	200	90
0·0025	210	134
0·005	218	212
0·01	268	304
0·02	314	406

The effects of phosphate concentration, fumarate, substrate concentration and pH are essentially the same as in T.T. cells.

No stimulation of deamination is produced by the addition of glucose or adenosine. On the contrary, in the absence of metal, the activity is invariably decreased in the presence of these two materials; in the presence of metal, however, no effect is obtained.

The method of freezing plays an important rôle in the response of activity to addition of metal. Slow freezing at —2° C. is more effective (i.e. produces a greater metal response) than fast freezing at —15° C. Moreover, the more dilute the suspension during freezing, the lower the basic activity and the greater effect of metal. Thick suspensions (more than 5 mg. dry wt. per ml.) frozen at —15° C. often show no metal requirement whatsoever. Stimulation by glucose and adenosine, however, disappears regardless of the freezing treatment employed.

DISCUSSION.

Hitherto, the requirement of aspartase for divalent metals has been unsuspected. This is probably due firstly to the fact that workers have generally used higher concentrations of aspartate, under which conditions the metal dependence is not evident, and secondly, because the organism of choice has usually been *E. coli*, a bacterium which, even in low substrate concentrations, appears to be less dependent on metal than *Proteus* or *A. aerogenes*. This seems to be due to some essential difference in the make-up of the cell, since cell-free preparations of aspartase from all three organisms exhibit similar metal requirements at low substrate concentrations (unpublished results). According to Jacobsohn and Pereira (1935), the presence of Mg^{++} shifts the equilibrium of aspartase in favour of deamination. This is unlikely to be the explanation for the stimulation of activity observed in this paper. (K37° C. for aspartase in the absence of Mg^{++} is *ca.* 130 (Jacobsohn and Tapadinhas, 1935), and allowing for the conversion of fumarate to malate, deamination of 0·0025 M L-aspartate is about 95 p.c. in favour of deamination. Yet strong metal stimulation is observed at the ten-minute interval when less than 1 p.c. of aspartate has been deaminated in its absence.

What is the action of the metal in the deamination reaction? Virtanen and Louhivuori (1947) suggest, without giving experimental details, that the shift of equilibrium towards deamination on the addition of Mg^{++} is due to precipitation of ammonia as a magnesium-ammonium-phosphate complex. Be that as it may, a similar explanation for the rate stimulation is considered untenable for the following reasons:

1. As already discussed, stimulation occurs when no significant amount of ammonia has been generated in the absence of metal.

2. Stimulation occurs equally with cobalt at a concentration one-hundredth that required for magnesium. Furthermore, a number of metals which are not inhibitory, but are equally as likely to form insoluble phosphate complexes have no action on the deamination. It will also be remembered that an increase in 2–5 times the optimal concentration of metal, while not inhibiting the reaction, fails to stimulate further, although on the above hypothesis more ammonia should be removed.
3. Added ammonia does not significantly affect the initial stages of the reaction in the presence or absence of stimulating metal.

The metal, therefore, must have some other function in the reaction. It is known that amino acids, including aspartic acid, will form complexes with divalent metals, and, furthermore, that the relative stability of metal complexes is usually $Co^{++} > Mn^{++} > Mg^{++}$ (Albert, 1950, 1952). A function of divalent metal in binding substrate to enzyme protein has also been suggested (see Lehninger, 1950 for references). The relaxation of metal dependence with increasing substrate concentration could be explained on such a basis.

The mechanism of stimulation by KCl and NaCl may be different, however, since it is difficult to see how Na^+ and K^+ could simulate the action of the divalent ions. There was strong evidence that the effects of these salts were not due to contamination with divalent metals. The elucidation of this point must await further investigation.

Gale in 1938 postulated the existence of two distinct enzymes, Aspartase I, insensitive to toluene and unaffected by adenosine, and Aspartase II, stimulated by adenosine and inhibited by toluene. The relationship of the T.T. cell activity to the "whole cell" activity is the subject of present research and will not be discussed here. However, Gale's criteria for the two enzymes must be criticized. That activity left after toluene treatment was termed Aspartase I, while the difference between this and the total activity of whole cells with adenosine represented Aspartase II. Now, the aerobic activity (or anaerobic activity with adenosine) of whole cells is essentially linear, while that of T.T. cells falls with time so that the ratio of whole cell activity to T.T. cell activity increases as the reaction proceeds. The presence or absence of metal may also vary the activity of T.T. cells while not affecting significantly that of whole cells.

Moreover, the substrate concentration used is critical. As has been shown, aspartase activity of T.T. cells against substrate concentration obeys the Lineweaver-Burk equation. However, the activity of whole cells is increased relatively little by similar increases in aspartate (Table 13). At low substrate concentrations, T.T. cell activity is lower than whole cell activity, but above *ca* 0·01 M aspartate, the T.T. cell activity exceeds that of the whole cells. With aspartate concentrations in the order of 0·1 M, T.T. cell activities of more than double the rate with those of whole cells have been obtained.

The results obtained with T.T. cells are consistent with the view that such treatment increases the permeability of the cell wall. Metal stimulation and fumarate and phosphate inhibition are not found with whole cells (unpublished results). Furthermore, the effect of substrate concentration on activity is small in whole cells as compared with T.T. organisms. This suggests that the entry and removal of these materials are restricted in the freshly prepared untreated cell.

In addition to (or concurrent with) this increase in permeability, there is a general disorganization of the enzymatic mosaic within the cell. While certain individual enzymes can still be demonstrated, for example urease, fumarase and L-amino acid oxidase, fermentation and oxidation generally are almost completely abolished. This undoubtedly explains the absence of stimulation by glucose, adenosine, etc.

The stabilizing effect of phosphate remains unexplained—its effect may be partly osmotic since other salts also stabilize, but much less effectively—but it is probable that the extreme lability of the enzyme in its absence is again brought about by the disorganization of cell structure effected by the toluene treatment. The general similarity between the activity in T.T. and frozen and thawed cells indicates that the latter treatment produces similar physico-chemical changes in the make-up of the cell. Its effect, however, is somewhat less drastic on the enzymatic content of the cell; for instance, a number of dehydrogenases remain active in frozen and thawed cells, but are totally absent after toluene treatment.

SUMMARY.

Aspartase activity of *Proteus* cells treated with toluene (T.T. cells) is stimulated by Mg^{++}, Mn^{++} or C^{++} at substrate concentrations below 0·01 M only. High concentrations of KCl or NaCl duplicate the effect of the divalent metals in phosphate but not in borate.

The enzyme in T.T. cells is unstable in distilled water, but is stabilized by phosphate at near neutral pH. At alkaline pH values, the enzyme is unstable even in phosphate.

The optimum pH of the reaction is between 7·1 and 7·5, depending on the metal used to stimulate.

The Michaelis-Menten constant for aspartase is of the order of 8×10^{-3} M in phosphate.

The deamination of aspartate is competitively inhibited by fumarate.

Aspartase activity in frozen and thawed cells exhibits many of the characteristics of that in T.T. cells.

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STUDIES ON THE PATHOGENICITY FOR ADULT MICE OF A VIRUS ISOLATED FROM A CASE OF PLEURODYNIA

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In a previous paper (Atkinson, Dineen and Robertson, 1951), the pathogenicity of an infant mouse virus, designated as PL.5, was demonstrated for adult mice. Further studies of the activities of this virus and of the natural history of the disease processes produced in adult mice have been made. It is the purpose of this paper to record these more detailed observations.

Pappenheimer (1951) reported the occurrence of pancreatic lesions in adult mice using the Conn.5 strain of the Coxsackie group of viruses. He described lesions in the pancreas, liver, spleen, abdominal fat and thymus after inoculation of the virus by various routes, but significant and permanent changes were noted in the pancreas alone, whatever the route of entry of the virus.

Our observations show that a similar limited tissue tropism exists for the PL.5 strain and that marked similarity exists between the lesions produced in the pancreas by our PL.5 and Pappenheimer's Conn.5 virus. Like Pappenheimer, we observed the pancreatic lesions independently in adult mice during the course of experiments on the production of immune serum. In the earlier stages of this work inoculation of adult mice by the cerebral route with first and second passage brain suspensions failed to produce disease or pancreatic lesions. However, when considerable amounts of tenth passage infant muscle suspension were inoculated intraperitoneally into adult mice for the production of antisera, disease became evident and the pancreatic lesions were first observed.

MATERIALS AND METHODS.

Preparation of virus suspensions.

From infant mouse muscle material. Moribund animals from the 10th passage of the PL.5 virus in infant mice were decapitated and both fore and hind limbs harvested and stored at —30° C. until required.

The limbs were ground with alundum in a mortar and homogenized in peptone broth to form a 15 p.c. suspension. After centrifugation at 10° C. for 30 minutes at 3,000 r.p.m., the supernatant fluid was transferred to a sterile 1 oz. McCartney bottle and 5 mg. of strepto-

[1] This work was carried out under the aegis of the South Australian Advisory Committee on Poliomyelitis with the aid of a grant from the Government of South Australia.

mycin and 500 units of penicillin added per ml. of suspension. After bacteriological sterility had been demonstrated, the preparation was dispensed with aseptic precautions into 1 ml. sterile ampoules and stored at —30° C. until required.

From adult mouse tissue. Animals were anaesthetized and exsanguinated by cardiac puncture 5 days after intraperitoneal inoculation of infant muscle suspension and the pancreas removed. A 15 p.c. pancreas suspension was prepared in peptone broth and centrifuged at 10° C. for 30 minutes at 3,000 r.p.m. Four hundred units of penicillin and 2 mg. of streptomycin were then added per ml. of supernatant. After demonstration of bacteriological sterility the suspension was ready for subinoculation.

Inocula were prepared from brain, eviscerated torso and limbs, in a similar manner, except that 5 mg. of streptomycin and 500 units of penicillin per ml. were added to preparations from eviscerated torso and limb material.

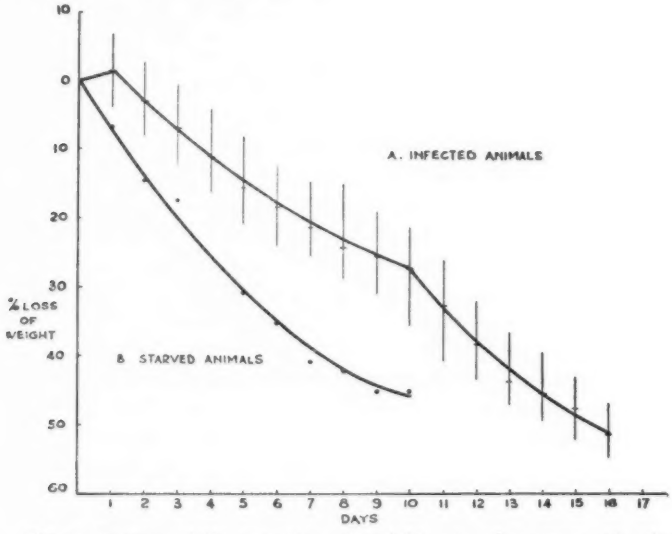

Fig. 1. Average daily p.c. loss of weight. A. of a group of mice following intraperitoneal inoculation of PL.5 virus. The upper ordinate represents the minimum p.c. loss (a weight gain occurred for the first 2 days following inoculation) and the lower ordinate the maximum p.c. loss. B. of a group of starved animals.

EXPERIMENTAL.

Signs of infection.

Following an intraperitoneal injection of 0·2 ml. of infected infant muscle suspension in 8 weeks' old male mice, the first obvious sign of infection was a loss of body weight. This frequently occurred within 24 hours, and invariably within 48 hours. During the course of infection the animals became hunched and weak, with ruffled fur, and ultimately died between the 13th and 18th days after injection. Of fifteen animals, only one survived for a longer period

and lived for 10 weeks. Signs such as flaccid paralysis and tremors, which were features in infant mice, were not associated with PL.5 virus infection in adult mice.

The average daily loss of weight for a series of fifteen 8 weeks' old animals, and the individual percentile loss of weight at death, following an intraperitoneal injection of 0·2 ml. of infant muscle suspension were determined.

For comparison, fifteen adult male mice were starved. Their average daily loss of weight, together with those of the infected animals are represented in Fig. 1.

The maximum average daily loss of weight occurred towards the culmination of infection on the 11th, 12th and 13th days and may be partly representative of the effects of loss of pancreatic secretion from the duodenum. The faeces were generally dry, although on occasions the presence of partially unformed faeces containing undigested fat was observed.

The percentile loss of weight at death for the starved and the infected animals varied between 30 p.c. and 50 p.c. of the initial body weight. The mean for both groups was 46 p.c.

Distribution of virus in adult mouse tissues.

Following the intraperitoneal inoculation of adult mice with 10th passage infant mouse muscle suspension, virus was recovered from the pancreas, limb muscle and eviscerated torso.

TABLE 1.

Virus distribution in adult tissues.

Inoculum	Litter	Days after inoculation 1	2	3	4	5	6	7	8	9	10	11	12	13	14	Average survival time
Brain Suspension	1	*1/7	1/7	1/7	1/7	1/7	1/7	1/7	1/7	1/7	1/7	1/7	1/7	1/7	1/7	
	2	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	
Pancreas Suspension	1	0/6	0/6	1/6	3/6	5/6	6/6									4·5 days
	2	*1/8	1/8	3/8	5/8	6/8	8/8									4·7 days
Eviscerated Torso Suspension	1	0/6	0/6	0/6	0/6	1/6	1/6	2/6	6/6							7·3 days
	2	0/7	*1/7	1/7	1/7	1/7	2/7	4/7	6/7	7/7						7·5 days
Muscle Suspension	1	0/6	0/6	0/6	1/6	1/6	3/6	5/6	6/6							6·3 days
	2	0/5	0/5	0/5	0/5	1/5	3/5	4/5	4/5	4/5	4/5	4/5	4/5	4/5	†4/5	6·0 days

Fractions in the Table represent the number animals dead/the number in the litter.

* Non-specific death. † Survived but stunted.

In an experiment to demonstrate the same tissue distribution of virus in adult mice following the injection of infected pancreas suspension from the adult host, ten animals were inoculated intraperitoneally with 3rd passage pancreatic tissue. Extracts were prepared from pooled pancreas, limb muscles, eviscerated torsos and brains harvested from the adult mice when they were

moribund and inoculated into infant mice by the peritoneal route. Infectivity titrations of pancreas, limb or torso were not performed but a relative estimate of virus concentration was obtained from the average survival times of litters inoculated with equivalent amounts of the tissues under investigation. For each, two litters were used. Those inoculated with pancreatic extract lived for an average of 4·5 days; those with limb extract lived for an average of 6·0 days and those with torso extract lived for an average of 7·5 days. None of the infants inoculated with brain suspension became ill or showed any signs of infection. The actual survival times are given in Table 1. From these results virus concentration appeared greatest in the pancreas.

Viraemia in adult mice.

Histologically there was no evidence of myositis or of pathological change in any tissues other than the pancreas and the liver. The infectivity of torso and muscle tissue from the limbs therefore suggested that a sustained or a transient viraemia in moribund animals occurred. To investigate this possibility a series of litters was injected with blood specimens collected from adult mice by cardiac puncture at varying intervals after inoculation.

Thirty-four 8 weeks' old mice were injected intraperitoneally with 0·1 ml. of a 10 p.c. infant mouse muscle suspension (10th passage). The animals were bled by cardiac puncture at arbitrarily selected intervals after injection and the blood heparinized. Two animals were sacrificed at each interval and the specimens pooled. The blood specimens were then tested for the presence of virus by injection of 0·2 ml. intraperitoneally into 1 day old infant mice. Some typical results are given in Table 2.

TABLE 2.

Comparative virus levels in the blood.

Period after injection when blood collected	Days after inoculation											
	1	2	3	4	5	6	7	8	9	10	11	12
3 hours	1/7	1/7	2/7	5/7	6/7	7/7						
6 hours	2/10	2/10	2/10	5/10	6/10	7/10	*9/10					
9 hours	0/6	0/6	0/6	2/6	3/6	6/6						
12 hours	1/8	1/8	1/8	5/8	7/8	8/8						
15 hours	0/8	0/8	1/8	3/8	6/8	6/8	6/8	*7/8				
18 hours	0/8	0/8	1/8	3/8	3/8	7/8	8/8					
24 hours	0/7	1/7	1/7	4/7	7/7							
30 hours	0/7	0/7	0/7	5/7	*6/7							
36 hours	0/5	0/5	3/5	5/5								
48 hours	0/6	0/6	6/6									
72 hours	0/6	0/6	0/6	6/6								
4 days	0/8	0/8	3/8	4/8	4/8	4/8	4/8	4/8	7/8	8/8		
5 days	1/6	1/6	2/6	2/6	2/6	2/6	2/6	2/6	2/6	5/6	6/6	
6 days	0/6	0/6	0/6	0/6	0/6	0/6	0/6	2/6	4/6	6/6		
8 days	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6

Fractions in the Table represent the number animals dead/the number in the litter.
* Survived but stunted.

From the relatively short survival time for the litter inoculated with the blood specimen collected from the animals sacrificed 48 hours after injection it was apparent that the maximum concentration of virus occurred in the blood stream between 36 and 72 hours following intraperitoneal injection. Virus was not detectable in the blood of infected animals by the 8th day.

Peritoneal lavage following intraperitoneal inoculation.

The possibility that the viraemia might be due to continued absorption of virus from the peritoneal cavity was investigated on the basis of the following experiment involving peritoneal lavage.

Eight 6 weeks' old male mice were injected intraperitoneally with 0·2 ml. of a 10 p.c. suspension of infected infant muscle. At daily intervals 2 ml. of sterile saline were injected into the peritoneal cavity and the animal exsanguinated under aether anaesthesia. The peritoneal exudate, supplemented with saline, was harvested and 200 units of penicillin and 2 mg. streptomycin added per ml. of fluid. After standing at room temperature for 30 minutes the "peritoneal fluid" was inoculated intraperitoneally (0·01 ml.) into day-old infant mice. Results are given in Table 3.

TABLE 3.

Comparative virus levels in the peritoneum following intraperitoneal inoculation.

Period between inoculation and lavage	Days after inoculation													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
24 hours	*1/8	1/8	3/8	8/8										
48 hours	0/8	1/8	8/8											
72 hours	0/6	0/6	3/6	6/6										
96 hours	0/6	0/6	1/6	5/6	6/6									
5 days	0/5	0/5	0/5	3/5	5/5									
6 days	*2/7	2/7	3/7	5/7	6/7	6/7	6/7	7/7						
7 days	0/6	0/6	1/6	1/6	3/6	3/6	3/6	3/6	3/6	4/6	4/6	4/6	4/6	4/6
8 days	0/5	0/5	0/5	0/5	0/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5

Fractions in the Table represent the number animals dead/the number in the litter.

* Non-specific deaths.

Table 3 shows that a decrease in the concentration of virus in the peritoneal cavity occurred. Nevertheless, the detection of virus in the "peritoneal fluid" at seven and perhaps eight days after inoculation indicated that absorption from this cavity may have occurred during the course of the viraemia.

Peritoneal lavage following intravenous inoculation.

In order to determine if the pancreas might release infective particles directly into the peritoneum, lavage of this cavity was performed after intravenous inoculation of the virus.

After centrifugation at 15,000 r.p.m., 0·1 ml. of a 10 p.c. infant muscle suspension was inoculated intravenously into four 6 weeks' old male mice. Lavage of the peritoneal cavity was performed on the 2nd, 3rd, 5th and 10th days in a manner similar to that adopted following intraperitoneal inoculation, and three randomized pools of one day old mice were inoculated intraperitoneally with 0·01 ml. of the "peritoneal fluid" harvested on the appropriate day.

TABLE 4.

Comparative virus levels in the peritoneum following intravenous inoculation.

Period between inoculation and lavage	Litter	Days after inoculation 1	2	3	4	5	6	7	8	9	10	11	12	13	14
2 days	1	0/7	1/7	6/7	6/7	6/7	6/7	6/7	6/7	7/7					
	2	0/7	0/7	5/7	6/7	6/7	6/7	7/7							
	3	0/7	0/7	3/7	4/7	4/7	5/7	6/7	6/7	7/7					
3 days	1	0/7	1/7	2/7	4/7	6/7	6/7	6/7	6/7	6/7	6/7	6/7	6/7	6/7	6/7
	2	0/7	0/7	4/7	4/7	5/7	5/7	6/7	6/7	6/7	6/7	6/7	6/7	6/7	6/7
	3	0/7	1/7	4/7	4/7	5/7	5/7	5/7	6/7	6/7	6/7	6/7	6/7	6/7	6/7
5 days	1	0/7	0/7	0/7	0/7	0/7	0/7	0/7	1/7	1/7	1/7	1/7	1/7	1/7	1/7
	2	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	1/7	1/7	1/7	1/7	1/7
	3	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7
10 days	1	0/7	0/7	2/7	2/7	2/7	2/7	2/7	2/7	2/7	2/7	2/7	2/7	2/7	2/7
	2	0/7	0/7	1/7	1/7	1/7	1/7	2/7	2/7	2/7	2/7	2/7	2/7	2/7	2/7
	3	*1/7	1/7	2/7	2/7	2/7	2/7	2/7	2/7	2/7	2/7	2/7	2/7	2/7	2/7

Fractions in the Table represent the number animals dead/the number in the litter.

* Non-specific death.

The results given in Table 4 indicate a gradual decrease in the virus content of the peritoneal fluid following intravenous inoculation. The discrepancy in the figures for the 10 day animal is consistent with the appearance of the lesions seen in the pancreas in this animal in that the changes appeared to be less advanced than those in the 5 day animal.

Microscopic findings in the pancreas.

The earliest lesion seen in the pancreas was a scanty infiltration of the capsular and interlobular fibrous tissues of the gland by polymorphonuclears and a few mononuclear cells. This change occurred between fifteen and eighteen hours after injecting the virus into the peritoneal cavity. Considerable increase in the numbers of inflammatory cells was seen in the next eighteen hours and there was an increasing oedema of the fibrous tissues with wide separation of the lobules. Part of this latter appearance seemed also to be due to shrinkage of the acinar cells which underwent severe degenerative changes by the end of the first 48 hours, few cells at this stage appearing normal. Degenerative changes affected the cytoplasmic contents a little sooner than the nuclei which, however, soon showed a preponderance of pyknotic forms. The cytoplasmic

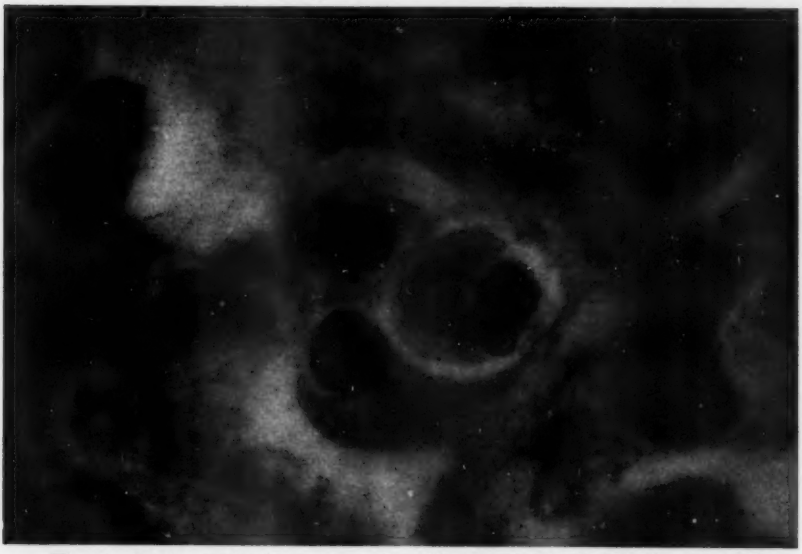

Fig. 2. In the centre is a group of medium-sized spherical bodies, partly laminated and entwined by basophilic threads of apparently similar material to that in the bodies. The rest of the degenerating cell is not seen in this section. (1,000 ×)

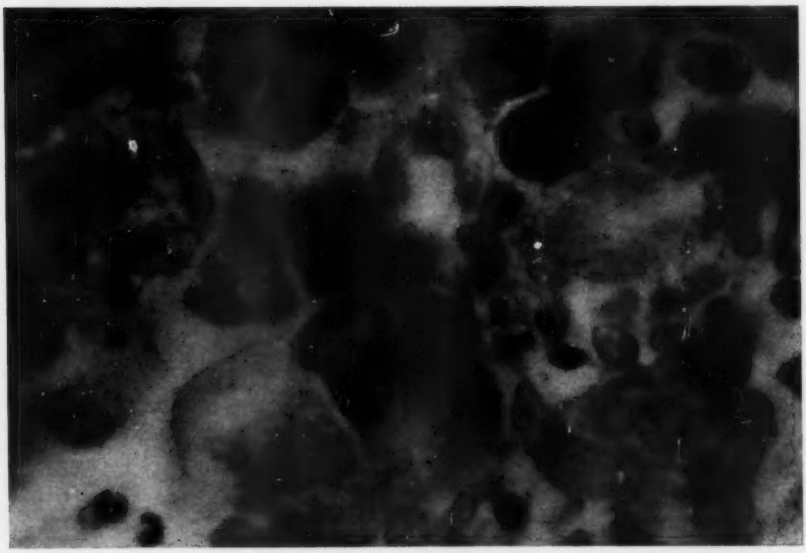

Fig. 3. Showing loss of normal acinar cell structure, a few inflammatory cells and fibroblasts. In the centre, a degenerating epithelial cell contains a large, partly-laminated, basophilic, spherical body. (840 ×)

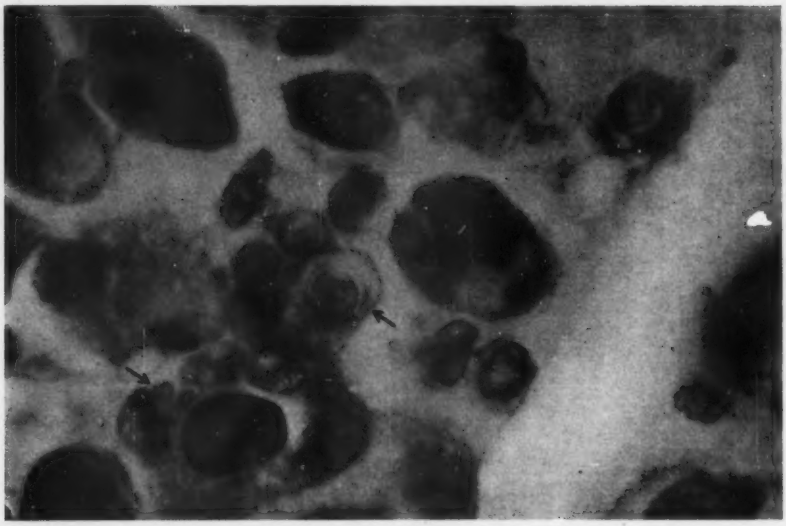

Fig. 4. The central arrow indicates a loosely laminated spherical body and the arrow on the left a much smaller one, along with others less distinctly seen, in the peripheral part of a degenerating cell. (1,000 ×)

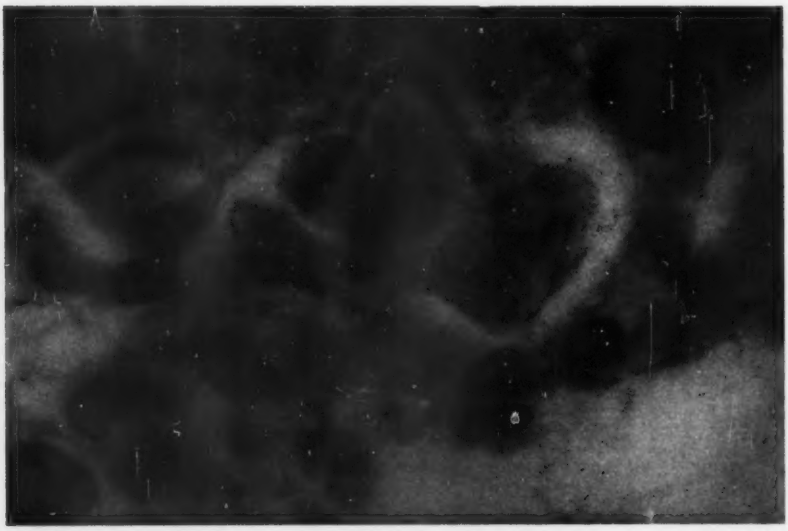

Fig. 5. Another group of entwined medium-sized spherical bodies. In the lower left-hand corner is a deeply basophilic laminated spherule of larger size. Fibroblasts can be seen amongst the degenerating acinar cells. (1,000 ×)

degenerative changes showed a variety of forms, the most peculiar of which occurred in the basophilic portions at the bases of the cells. Numerous abnormal structures of roughly spherical appearance were seen in many cells at this stage. They varied widely in number and size. In some cells several small round bodies occurred in clusters (Figs. 2, 5) while in others there was a single circular structure two to three times the diameter of a normal acinar cell nucleus (Fig. 3). These larger structures usually had the appearance of possessing a laminated outer covering and, in some, a remarkable onion-skin effect was produced (Fig. 4). The smallest, which were usually multiple (Fig. 4), were approximately the size of, or smaller than, a red cell. Occasionally in association with these abnormal structures there were small vacuoles in the adjacent cytoplasm which in other cells could be seen unrelated to the spherical bodies. In addition to the formation of spherules the development of a lake of uniform, homogeneous, basophilic material or the formation of fine basophilic threads running throughout the cell, was observed. Sometimes these threads gave the impression that by further coiling and shrinkage the formation of a laminated spherule might have resulted. In common with the spherules, these other abnormal structures seemed to result from degeneration of the proximal cytoplasmic contents. The nuclei in most of the cells containing these structures were the most pyknotic of all the nuclei in the pancreas at this stage of the disease and generally appeared to be separate from the abnormal cytoplasmic bodies. The spherical bodies were not phloxinophyllic, they stained red with the Unna-Pappenheim stain and occasionally purple using the Feulgen reaction. Few cells at this stage contained zymogen granules and, in nearly all, the secretory part of the cell was occupied by irregular pale-staining eosinophilic granular material. In large areas the acinar cells were almost completely disorganized. Where this occurred, polymorphonuclear infiltration of the lobule was a prominent feature.

At the end of 72 hours the gland showed a more or less uniform eosinophilia and few cells contained basophilic material. Most of the cells were without nuclei and the cell membranes enclosed coarse eosinophilic granules or fine eosinophilic threads and leucocytes. Fibroblastic proliferation was now a marked feature of the lesion and patchy lymphocytic infiltration occurred throughout the gland. The intensity of the polymorphonuclear infiltration of the interlobular and intralobular areas was undiminished and oedema of the interstitial tissues together with shrinkage of the acinar cells further accentuated the separation of the lobules.

On the fourth day a very considerable fibroblastic proliferation had occurred and necrotic eosinophilic remnants of the acinar cells were patchily dispersed among the fibrous tissue. At the end of five days the gland was almost completely replaced by fibroblasts which surrounded apparently intact Langerhan's islets and pancreatic ducts. These structures showed no obvious destruc-

tive changes except that in the later stages (8–12 days) the ducts were often dilated.

From five to twelve days progressive fibrosis and shrinkage of the pancreas occurred and by about the ninth day large areas showed fat-cell infiltration. Between the fourth and seventh day acini were not seen but one or two were observed in specimens from the seventh day onwards and in a twelve day specimen appeared structurally normal and fairly numerous.

DISCUSSION.

After injection of the PL.5 virus by the peritoneal route into adult mice, virus particles were rapidly absorbed into the blood-stream and disseminated throughout the body. However, permanent lesions were observed in no tissue other than the pancreas where changes were regularly seen within 18 hours after inoculation.

In two or three days following the rapid degeneration of the pancreatic acini the animals became hunched and weak. These changes were considered to be due either to the probability of the animals not eating or to the effects of loss of pancreatic enzymes from the digestive tract. That inanition was an important factor was borne out by the observation of similar changes in normal animals after three days' starvation. To assess accurately the food intake in the infected animals was impossible but, as they lived longer, on average, than normal starved animals, this indicated that they took some nourishment during the course of the infection. The presence of intact acini in some 10–12 day animals suggested that in those which survived for longer periods some pancreatic secretion was present. A curious feature was the failure to observe structurally intact acini between the third and seventh day of the infection, but the presence of acinar tissue from the seventh day onwards suggested that some cells had escaped complete destruction and had become actively secreting units again.

The maximum concentration of virus occurred in the blood-stream between 36 and 72 hours after intraperitoneal injection. Thereafter the level progressively decreased until the eighth day, after which virus was not detected. Concurrently with the viraemia, virus was isolated from the peritoneal cavity over the same period, which suggested that the peritoneal cavity may have been the source of virus for the blood-stream. In addition, the pancreas may have been the source of a continued supply of virus to the peritoneal cavity.

Experiments designed to support these contentions showed that virus entered the peritoneal cavity after intravenous inoculation but was recovered only up to five days following injection, while the pancreas contained virus for a much longer period. Probably therefore, the pancreas was a more potent source of virus for the blood-stream than the peritoneal cavity. Further, the occurrence of the highest concentration of virus in the blood-stream between 48 and 72

hours may have manifested virus multiplication in the early stages of infection in the pancreas.

In the early stages of acinar cell degeneration unusual structures were observed in the cytoplasm of some of the affected cells. On morphological grounds the localization of spherical structures in the proximal portions of cells and the fact that a precursor stage in their formation appeared to be related to condensations out of the basophilic cytoplasm, made a cytoplasmic origin appear most probable. The presence in the affected cells of intact or degenerating nuclei which did not have an anatomical relationship to the bodies suggested that they were probably not of nuclear origin. On the evidence obtained so far a definite opinion on the exact mode of development of these transient features of the acinar cell necrosis cannot be expressed and, while they appeared to be products of the degenerating cytoplasm, the possibility of their being virus inclusion bodies cannot be dismissed.

SUMMARY.

Further observations on the natural history of infection produced by a virus, designated as PL.5, in adult mice are reported.

Loss of weight in infected animals has been correlated with that in a group of starved animals. The percentile loss of weight at death, which in the starved animals occurred approximately 6 days before death in infected animals, varied between 30 p.c. and 50 p.c. in both groups.

A rough quantitative estimation of the virus content of various tissues in infected animals has been made. The greatest concentration was found in the pancreas.

The highest concentration of virus in the blood was between 36 and 72 hours after intraperitoneal inoculation. Thereafter the level decreased until the eighth day when virus was not detected.

Estimation of the virus content of the peritoneal cavity after intravenous inoculation showed a similar decrease with an insignificant amount present on the fifth day. The pancreas contained virus for a longer period and is considered a more potent source of virus for the blood-stream than the peritoneum.

Lesions in the pancreas in the early stages of the infection have been described. Attention has been drawn to peculiar spherical bodies in the degenerating cells which are thought to be products of cytoplasmic breakdown.

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THE PREPARATION AND PROPERTIES OF AN INHIBITOR OF INFLUENZA VIRUS HAEMAGGLUTINATION FROM HUMAN MECONIUM

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Since Francis (1947) described the presence of a substance in normal serum inhibiting haemagglutination by influenza virus (LEE) previously heated at 56° C. for 30 minutes, a number of other substances have been shown to have this inhibitory quality. Burnet (1948a) first showed that the fluid from some human ovarian cysts inhibited suitably treated influenza viruses. Purified blood group substance obtained from ovarian cyst fluid (Morgan and King, 1943) was also shown to act as an inhibitor (Burnet, 1948a). Gottschalk and Lind (1949) showed that the inhibitory quality of egg-white was carried in the ovomucin fraction of this substance. Anderson (1950) examined the tissues of a number of animals and found that the mucin secreting glands and tissues in particular yield extracts with marked inhibitory properties. Recently other substances have been described as inhibitors of virus haemagglutination. Thus saliva (Francis and Minuse, 1948), urine (Tamm and Horsfall, 1950) and milk (Lanni, Lanni and Beard, 1949) have been shown to possess in varying degree this quality.

Considerable use has been made of crude and semi-purified preparations of inhibitors from various sources in studying the enzymic action of influenza viruses and the nature of the enzyme-substrate relationship (see review by Burnet, 1951). For some time now, however, it has been apparent that for further studies along these lines a readily available and reproducible supply of a relatively pure inhibitor substance is needed. Purified urinary inhibitor fulfils most of the requirements in this respect but is relatively sensitive to destruction by heat. The provision of another purified substance with inhibitor quality would make available a second substrate for enzyme studies with the receptor destroying enzyme of the influenza viruses.

The report of Rapoport and Buchanan (1950, 1951) on the finding that a considerable part of human meconium is carbohydrate in nature and that they could readily obtain human blood group mucoid from this substance caused

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Sir Macfarlane Burnet, F.R.S., to suggest to one of us that we examine human meconium for virus inhibitor. This paper describes the partial purification and general biological properties of the inhibitor of influenza virus haemagglutination found in meconium from new-born human infants.

Materials and Methods.

Biological reagents and methods.

Viruses. The following strains were used in the form of infected allantoic fluid harvested from embryos inoculated at the age of 11 days and incubated for 40 hours at 35° C.

Influenza A—egg adapted sub-strain of WS (WSE), PR 8, Melbourne (MEL), FMI and Swine

Influenza B—LEE and MIL.B.

Indicator viruses (Stone, 1949a) denoted by the prefix H = heated or T = treated. T.MEL, T.PR 8 T.LEE, T.FMI, T.MIL.B and T.Swine were prepared by adding 0·5 ml. 20 p.c. sodium citrate and 0·5 ml. borate buffer pH 8·4 to 10 ml. of infected allantoic fluid and heating the mixture at 56° C. for 30 minutes.

H.WSE. Infected allantoic fluid heated at 52° C. for 30 minutes.

Physiological saline contains 0·9 p.c. sodium chloride in distilled water.

Calcium acetate saline. Sodium acetate 12·3 gm. sodium chloride 5·0 gm. calcium chloride anhydrous 1·0 gm. 2 N acetic acid 1·3 ml. in 1 litre of distilled water. It can be sterilized by autoclaving after which the pH should be about 6·2.

Borate buffer. Equal parts of M/20 borax and M/5 boric acid solution pH 8·5.

Receptor destroying enzyme (RDE) of *V. cholerae.* Purified enzyme prepared by the method of Ada and French (1950).

Titration of inhibitor. Serial twofold dilutions of inhibitor were prepared in 0·25 ml. of physiological saline. An equal volume of a dilution containing five agglutinating doses of the appropriate indicator virus was added to each tube and the mixtures allowed to stand for 30 minutes at room temperature. A third volume of 1 p.c. suspension of washed fowl erythrocytes in physiological saline was then added and the test read by the pattern of haemagglutination after a further hour at room temperature. The end-point was the "+" degree of partial haemagglutination.

Titration of blood group substance. The quantitative methods recommended by Wiener (1943) were used. Dilutions of meconium in saline were incubated at room temperature with 8 agglutinin units of human serum for 15 minutes and then a volume of human red cells of the appropriate group added. The test was performed in tubes 7·5 cm. long by 1 cm. in diameter and the result read by the pattern of the cells in bottom of the tube settling at room temperature.

Chemical reagents.

Buffers used as diluting fluids.

pH 3 0·08 M glycine, 0·08 M NaCl, O·02 M NaCl, 0·02 M, HCl.
pH 4 0·08 M acetic acid, 0·02 M sodium acetate.
pH 5 0·03 M acetic acid, 0·07 M sodium acetate.
pH 6 0·05 M KH_2PO_4, 0·0056 M KOH.
pH 7 0·045 M KH_2PO_4, 0·27 M KOH.
pH 8 (a) 0·05 M KH_2PO_4, 0·047 M KOH.
(b) 0·0716 M veronal, 0·0284 M HCl.

pH 8·5 calcium borate buffer 0·06 M sodium borate, 0·04 M HCl, 0·02 M $CaCl_2$.
pH 10 0·062 M glycine, 0·062 M NaCl, 0·038 M NaOH.
pH 11 0·051 M glycine, 0·051 M NaCl, 0·049 M NaOH.
pH 12 0·045 M glycine, 0·045 M NaCl, 0·055 M NaOH.

Electrophoresis buffers. Potassium phosphate pH 7, ionic strength $\mu = 0{\cdot}2$; 0·091 M KH_2PO_4; 0·055 M KOH.

Electrophoresis: Electrophoresis was carried out at 2° C. in a Tiselius apparatus with Philpot-Svennson optical system (Holden, Ada and Pye, 1952). Analytical work was performed in an 11 ml. cell with long centre section while the standard two-section cell (11 ml.) was used for preparative work. Where only small samples were available a microcell (2 ml.) was used.

Collection of meconium.

Specimens of meconium were obtained from the obstetric departments of four large hospitals in the city of Melbourne. Sterile screw cap 4-oz. glass jars were supplied to the hospitals and the specimens of meconium collected from the sterile diapers into these jars during the first 24 hours after the baby was born. Specimens collected in this way were found to be nearly always free from bacteria cultivatable on a blood agar plate; an occasional specimen yielded a scanty growth of a white staphylococcus and a few specimens produced a growth of fungus. Specimens obtained during the second or subsequent days after birth were found to be contaminated with faecal bacteria and the inhibitor titre of these contaminated specimens was either much reduced or nil. The sterile specimens could be kept at room temperature without loss of titre and in view of this a collection of specimens was made from the hospitals on the average once a week.

EXPERIMENTAL.

Purification of the meconium inhibitor.

Fractionation with miscible solvents. Experiments were carried out applying the process of solvent fractionation to aqueous homogenates of the crude meconium. Methanol was used under various conditions of pH and temperature, and in the presence of the heavy metals barium and zinc (Cohn, 1950). Attempts were also made to fractionate with acetone (Askonas, 1951) under similar conditions. These methods were unsuccessful in effecting a purification. It was noted during these experiments that irreversible inactivation of the inhibitor occurred in the presence of barium salts

Treatment with chloroform. A considerable degree of purification was obtained by repeated shaking of the aqueous meconium either with chloroform alone or with mixtures of the latter with varying amounts of amyl alcohol (Sevag, 1934). This method, however, resulted in an overall recovery of less than 10 p.c. of the original inhibitor activity.

The use of phenol in the purification of the meconium inhibitor. McCrea (1951) has reported that the inhibitor isolated from sheep sub-maxilliary gland is insoluble in 90 p.c. phenol. Preliminary experiments showed that this property was shared by the meconium inhibitor and suggested a possible means of purification by removal of the phenol soluble constituents in meconium reported by Rapoport and Buchanan (1951). Early efforts in this direction were ham-

pered by the formation of intractable emulsions on the addition of 90 p.c. phenol to aqueous homogenates of meconium. To avoid this it was found necessary to dissolve the crude meconium in the phenol and to extract the inhibitor into an aqueous phase. The method described below was found to be the most satisfactory. All the procedures described were carried out at 5° C., except where otherwise stated.

Twenty specimens of meconium (average weight 10 gm. each) were homogenized by means of a Waring Blendor in 400 ml. of 90 p.c. phenol for 5 minutes. The phenol solution was gently shaken with 500 ml. of cold saturated sodium acetate solution. On centrifugation for two hours at 1,500 *g* the phenol and aqueous layers separated and the latter which contains the inhibitor was kept (vol. approx. 400 ml.). The last traces of phenol emulsion were removed from this solution by chilling at and centrifuging at —3° C. for 30 minutes at 2,500 *g*. Dissolved phenol and sodium acetate were removed from the supernatant by dialysis for forty-eight hours against two changes of excess distilled water. The clear solution thus obtained was dried from the frozen state *in vacuo* to give a pale yellow-green powder having an activity of 50,000 inhibitory units per mg. dry weight.

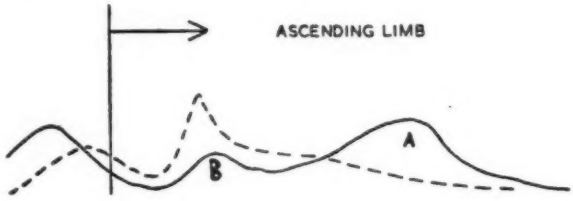
Fractionation with methanol. This material may be further purified by fractionation with methanol. The dry powder is dissolved in the pH4 ($\mu = 0{\cdot}1$) acetate buffer to give a 2 p.c. (w/v) solution. Methanol, previously chilled to —40° C., is added slowly with stirring until the concentration reaches 66 p.c. (v/v). The precipitate which forms may be adequately separated by decantation and is dissolved in a minimum amount of distilled water giving a green solution. Methanol is removed by dialysis for forty-eight hours against two changes of excess distilled water.

The green colour may be removed by absorption on to a zinc hydroxide precipitate as follows. The inhibitor solution is made 2N with respect to zinc by the addition of solid zinc sulphate. Twice normal sodium hydroxide is added in equal volume to precipitate all the zinc hydroxide. The precipitate is removed by centrifugation at 1,500 *g* in an angle head centrifuge and the de-colourized supernatant containing the inhibitor is freed of traces of zinc by dialysis against the sodium citrate buffer and then against several changes of distilled water. On drying this solution from the frozen state *in vacuo* a white powder is obtained with an activity of 80,000 inhibitor units per mg. dry weight. The overall yield of these steps is approximately 50 p.c.

Electrophoretic examination of the material obtained in this manner (Fig. 1) indicated a major group of components (A) with mobilities ranging approximately from 3×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} to $7{\cdot}5 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1}. A slower component (B) of approximate mobililty 1×10^{-5} cm.^{2} sec^{-1} volt^{-1} is also present. Treatment with RDE modifies the pattern considerably.

The simplest explanation of the change is that the mobility of much of A has been reduced to approximately that of B.

Attempt at electrophoretic separation of inhibitor. This was carried out on material prepared by methanol fractionation as described above. Since it was suspected that the inhibitory activity was associated with the larger part of "A" an electrophoretic separation of this was attempted. It was found that the faster component "A" carried 72 p.c. of the inhibitory activity and the slower component "B" carried only 28 p.c. Despite the small amount of material available, electrophoresis before and after RDE treatment of the electrophoretically separated fraction "A" indicated that the inhibitory fraction had been considerably purified. However, non-inhibitory fast components present in undetectable quantities in the chemically prepared samples were also concentrated to a point where they became detectable in the electrophoresis pattern (Fig. 2).



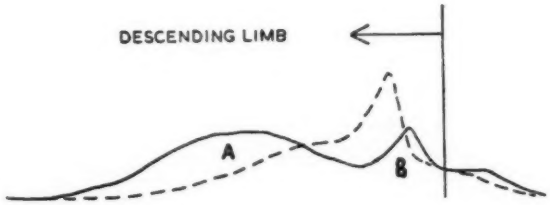


Fig. 1.

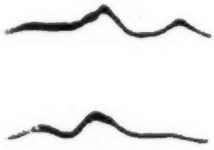


Fig. 2.

Fig. 1. Electrophoresis of phenol extracted inhibitor fractionated with methanol.
——— before treatment with RDE.
- - - - - - after treatment with RDE.
Phosphate buffer, pH 7, ionic strength, (μ) = 0·2. 11,600 seconds at 3·35 volt./cm.
The vertical line and arrow refer to the position of the initial boundary and the direction of migration.

Fig. 2. Electrophoresis of material obtained by electrophoretic separation of component A. (see Fig. 1).
Phosphate buffer, pH 7, μ = 0·2. 6,200 seconds at 3·25 volt./cm.

Blood group activity of meconium after fractionation. Details of the blood groups of the infants furnishing the meconium were obtained and a group of specimens from infants of blood Group A was subjected to the fractionation procedure, and the phenol fraction collected as well as the aqueous sodium acetate fraction. After dialysis an examination of the fractions for inhibitor and blood group substance activity was made. The inhibitor and blood group activity was distributed almost equally between the phenol and the aqueous fraction. Further extraction of the aqueous inhibitor fraction with phenol and shaking with chloroform failed to alter the titre of the blood group substance in this solution.

Biological properties of the inhibitor.

Effect of pH and heat. A volume of semi-purified meconium was mixed with four volumes of buffer and the mixtures were plunged into a boiling water bath for 1 minute. After rapidly cooling, the inhibitor titre was determined. Between pH 3·0 and 11·C no reduction in the titre occurred with this treatment; at pH 12·0 there was a 16-fold drop in titre. The pH of mixtures was tested after heating and found to be within 0·2 of a pH unit of the value for the buffer. Mixtures at pH 3·0 and 11·0 were heated in a water bath at 100° C. and samples withdrawn at different times and the inhibitor titres estimated. Table 1 sets out the results obtained.

TABLE 1.

Effect of heating purified meconium inhibitor at 100° C. in buffers.

Time at 100° C.	pH 3·0	pH 11·0	Saline
1 min.	800	800	800
2 min.	400	700	800
5 min.	100	600	800
10 min.	<100	300	800
20 min.	<100	100	800

These results show that at pH 3·0 and 100° C. 12 p.c. of inhibitor activity remained after five minutes and at pH 11·0 under similar conditions 75 p.c. remained after 5 minutes and 12 p.c. after 20 minutes. In unbuffered saline there was no evidence of destruction of inhibitor activity after 20 minutes at 100° C.

TABLE 2.

Titration of meconium inhibitor with 5 AD T.MEL virus and cells from different fowls.

Fowl No.	Titre	Fowl No.	Titre	Fowl No.	Titre	Fowl No.	Titre
733	1·5	750	7	751	12	766	16
752	1·5	717	8	742	12		
701	2·0	714	8	749	12	762	20
		735	8	754	14		
				728	14		

Anderson (1948) has described individual variation between fowl cells used in titrating the non-specific inhibitor in human serum. Fourteen fowls were bled into citrated saline and the erythrocytes collected and washed three times in saline. The cells were then packed firmly by centrifuging at 3,000 r.p.m. for 20 minutes and then made up as a 1 p.c. suspension in normal saline. These cells were used to titrate a solution of purified meconium inhibitor with results shown in Table 2. An initial dilution of 1/5,000 was made.

Using three different types of fowl cells, an experiment was made varying the time of contact of the inhibitor with the indicator virus before adding the red cells. A series of dilutions of meconium was made in sufficient quantities to distribute in 12 sets of tubes. To the first 3 sets the 3 different types of cells were added and then the indicator virus. To the second 3 sets the indicator was added and after 30 minutes the red cells. The third and fourth sets of dilutions were allowed 60 and 120 minutes' contact with the indicator virus respectively before the addition of red cells. Table 3 sets out the results obtained.

TABLE 3.

Results of varying time of contact of inhibitor with indicator before adding red cells.

Time in minutes	Fowl 752	Fowl 750	Fowl 762
0	0	0·5	3
30	1·5	7	20
60	2	14	35
120	4	28	100

It can be seen that with increasing time of contact between inhibitor and indicator virus in this experiment, the titre of the inhibitor was increased with each type of fowl red cells.

The action of virus enzyme and V. cholerae *RDE on the inhibitor.* Burnet (1948b), Stone (1949b) and more recently Fazekas de St.Groth (1952) have studied the dynamics of virus enzyme and *V. cholerae* RDE action on various inhibitors. This work has shown that each inhibitor has its characteristic pattern or inhibitor gradient.

The action of the various viruses and RDE was tested on purified meconium inhibitor in the following experiment. Using allantoic fluid virus no more than 48 hours old a preliminary determination was made of the minimum amount of virus necessary to reduce the titre of the inhibitor by at least 90 p.c. in 4 hours at 37° C. The test proper was then performed with these titrated viruses.

To each of four test tubes containing 0·75 ml. of a suitable dilution of the purified inhibitor, 0·25 ml. of virus dilution in saline and one drop of toluene were added to each tube. The tubes were stoppered and incubated in a water bath at 37° C. At 30, 60, 120 and 180 minutes after mixing a tube was removed from the water-bath, immersed in boiling water for 1 minute to destroy the virus

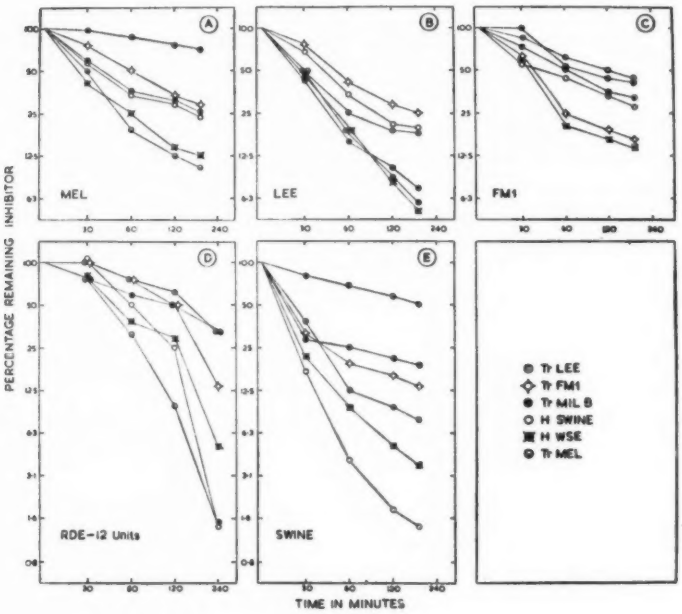


Fig. 3.

Fig. 3 A shows the action of active MEL virus on the inhibitor of the different indicator viruses.

Fig. 3 B shows the action of active LEE virus on the inhibitor of the different indicator viruses.

Fig. 3 C shows the action of active FMI virus on the inhibitor of the different indicator viruses.

Fig. 3 D shows the action of RDE on the inhibitor of the different indicator viruses.

Fig. 3 E shows the action of active Swine virus on the inhibitor of the different indicator viruses.

and then cooled. The residual inhibitor in each test tube was titrated with each of the indicator viruses, viz. T-MEL, T-LEE, T-FMI, H. Swine, H.WS. and T-MIL.B. A control tube of inhibitor in which the virus was replaced by saline was included in each case. In addition to the viruses, purified RDE was used. In this instance the reagents were diluted in calcium acetate saline pH 6·2. From the results of the titrations for inhibitor in the test and control tubes the percentage of residual inhibitor was calculated. Figs. 3a, b, c, d, and e show the results obtained. It can be seen that in each case the active virus reduces the titre of the inhibitor for its corresponding indicator to a level as low as, or lower than, for the other indicators.

The prolonged action of RDE on meconium was tested. Twelve units of RDE were allowed to act on meconium in the presence of toluene for 24 hours at 37° C. After heating in the boiling water-bath as above the inhibitor activity for the various indicators was determined. In every case excepting treated FMI, the residual inhibitor titre was below 1 p.c. of original. In the case of treated FMI 4·5 p.c. remained. This residual inhibitor was readily abolished by allowing about 150 agglutinating units of active FMI or 1,000 units of RDE to act for 150 minutes at 37° C.

The action of other enzymes on meconium inhibitor. Gottschalk and Lind (1949) found that the inhibitor in ovomucin was destroyed by the action of purified crystalline trypsin. The action of trypsin on purified meconium was tested by incubating at 37° C. for 24 hours a solution of meconium in calcium borate buffer (pH 8·5), with a small quantity of crystalline trypsin in the presence of toluene. Controls consisted of meconium without trypsin and meconium with trypsin and soya bean trypsin inhibitor. In each case the inhibitor titre was identical after 24 hours' incubation. The titre of the trypsin in the test was determined after the period of incubation by means of the photographic plate technique (Harrison, 1939) and found to be active at a dilution of 1/256.

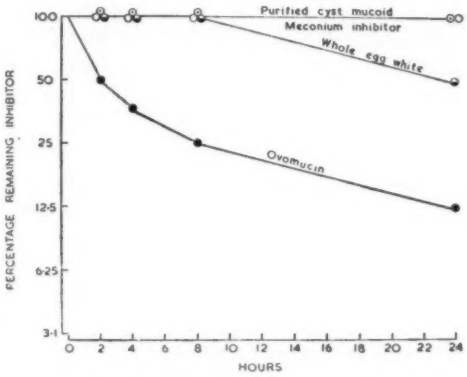


Fig. 4 showing the action of taka-diastase on purified cyst mucoid, meconium inhibitor, whole egg white and ovomucin.

Rimington (1950) has recorded the destruction of gonadotrophic hormone [which is also destroyed by influenza enzyme or RDE (Whitten, 1948)] by taka-diastase. Professor Rimington kindly made available a sample of the taka-diastase used in his experiments and a 1 p.c. solution of this was mixed with three different inhibitors, meconium, ovomucin and cyst mucoid and incubated at 37° C. for 24 hours in the presence of toluene. A control without enzyme preparation was included. Samples were withdrawn at various intervals from the test and control tubes and tested for residual inhibitor. The results are set out in Fig. 4, and show that meconium and cyst mucoid are unaffected by taka-diastase, whilst the ovomucin inhibitor titre undergoes a steady fall over 24 hours. The one tube fall with whole egg white between 8 and 24 hours was obtained in both experiments but is hardly significant.

DISCUSSION.

Our study of meconium from new-born infants confirms the observations of Rapoport and Buchanan (1951) concerning the presence of blood group substance in this material. In addition to the latter we have found that human meconium contains a substance carrying the properties of an inhibitor of haemagglutination by suitably treated influenza viruses. It has been our experience that this inhibitor can be regularly obtained in good yield. The product obtained, however, was not pure inhibitor, at least one of the contaminants being an undetermined amount of blood group substance. Further extraction with phenol did not remove this. It was not ascertained whether the blood group contaminant was still present in the electrophoretically separated material owing to the difficulty of obtaining sufficient meconium of the one blood group to prepare inhibitor for the experiment. Further experiments are being carried out in an attempt to free the inhibitor from blood group substance.

The biological properties of the inhibitor showed considerable stability under a variety of conditions. Thus, the inhibitor was relatively stable to temperatures of up to 100° C. over the range pH 3–11. Its relative insensitivity to shaking with chloroform is in contrast to the destruction by this treatment of egg white and milk inhibitors. It was noted, however, that the inactivation of the inhibitor by the presence of barium ions could not be reversed even by the addition of alkaline earth "de-ionising" reagents such as citrate. This accords with the experience gained in the study of the sputum inhibitor (Marmion, Curtain and Pye, 1953 and Curtain, Marmion and Pye, 1953) and the urinary inhibitor (Curtain, 1953). In both cases the barium causes inactivation and precipitation at quite low concentrations.

Biologically the meconium inhibitor is typical of other products of mucus secreting surfaces. Thus it is active against haemagglutination by treated virus to a high titre but is destroyed by suitable concentrations of active influenza viruses and the receptor destroying enzyme of *Vibrio cholerae*. Furthermore, as Fig. 4 shows, each virus has its own characteristic action reducing the titre for itself more than for other indicators.

The results of the experiments using erythrocytes from different fowls (Table 2) indicate that there is some degree of variation between fowls but not to nearly the same extent as was found for normal human serum inhibitor and heated LEE virus indicator where the titre with certain fowl cells was about 100 times greater than with other fowl cells (Anderson, 1948). The results of varying the time of contact of the inhibitor with the indicator virus shown in Table 3 are similar to the results obtained by Lanni and Beard (1949) with egg white inhibitor and by Tamm and Horsfall (1950) with urinary inhibitor.

The marked heat stability of this inhibitor over a wide pH range makes it a particularly useful reagent for studying interaction between the cell sur-

face, indicator virus and inhibitor and other related problems (Burnet and Edney, 1952; Burnet, 1951).

The experiments with taka-diastase showed that this substance had no RDE-like action on the meconium or the cyst mucoid. On the ovomucin a slow action could be demonstrated which was suggestive of the action of trypsin on ovomucin (Gottschalk and Lind, 1949). It was found that the sample of taka-diastase had a low titre of tryptic activity as determined by the photographic plate technique (Harrison, 1939). When taka-diastase was allowed to act on whole egg white, the inhibitor was not destroyed under the above conditions. It is known that whole egg white has a substantial anti-tryptic activity.

SUMMARY.

The purification and properties of an inhibitor of influenza virus haemagglutinin from human meconium are described.

The inhibitor can be partially purified by extraction of a phenol solution of crude meconium with saturated sodium acetate solution followed by low temperature methanol fractionation.

The inhibitor withstands boiling over a wide pH range and biologically resembles cyst mucoid inhibitor.

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BACK-RECOMBINATION OF INFLUENZA A STRAINS OBTAINED IN RECOMBINATION EXPERIMENTS

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In an earlier paper (Burnet and Lind, 1952) the isolation of two recombinants, M+ and WS—, from the products of the first growth cycle of a double infection with Melbourne (MEL) and WSE viruses in the allantoic cavity of developing eggs was described. The parent strains MEL and WSE could be clearly distinguished from one another by means of certain tests *in vitro* and *in vivo* and, by submitting the two recombinant strains to the same procedures, it was shown that each had derived certain characters from both the parent strains and, further, that each recombinant was the reciprocal of the other. Both new strains were stable and maintained their characteristics unaltered after several allantoic passages in eggs at limit infecting dilution. This paper deals with the results of further experiments in which a mixture of the two recombinant strains was used to initiate double infection in the allantoic cavity and the parent strains MEL and WSE isolated from the fluid containing the yield of the first cycle of virus growth.

MATERIALS AND METHODS.

Virus strains. Freshly harvested virus from the third allantoic passage, each at limit infecting dilution, of the two recombinant virus strains M + and WS— (Burnet and Lind, 1952) was used in these experiments. Details of the dosage will be given in the text.

Receptor destroying enzyme (RDE). Semi-purified receptor destroying enzyme of *V. cholerae* prepared according to the method of Ada and French (1950).

Ringer solution. NaCl 8·5 gm.: KCl 0·25 gm.: $CaCl_2$ 0·3 gm. in 1,000 ml. distilled water.

Normal saline. NaCl 8·5 gm. in 1,000 ml. distilled water.

One cycle allantoic fluids. The method used was essentially that developed by Cairns (1952) to analyse the products of one cycle of virus reproduction in the allantoic cavity, and was first used to study the products of double infection by Fraser and Burnet (1952). Embryonated eggs on the 11th or 12th day of development were prepared by drilling two holes as for allantoic inoculation and the surrounding area of the shell swabbed over with melted paraffin wax. As the volume of inoculum used was large, an equivalent amount of allantoic fluid was withdrawn before injection of the virus mixture. Following inoculation, the eggs were placed at 37° C. for 1 hour for virus adsorption to occur, after which 10,000 units of RDE were inoculated into the allantoic cavity to remove all virus adsorbed to surface receptors and to destroy any intact receptors which might adsorb virus liberated at

the end of the first reproductive cycle. RDE was allowed to act for one hour at 37° C. after which the allantoic cavity of each egg was drained by aspiration with a Pasteur pipette and washed out six times with warm Ringer solution. The fluid was finally replaced by 5 ml. warm Ringer containing 1 p.c. horse serum and incubation was continued until the 6th hour after infection. The allantoic content of the eggs which contained virus liberated after the first cycle of multiplication was then harvested.

In order to determine what virus types were present in these allantoic fluids limit dilution titration methods were used. Tenfold dilutions of the fluids were prepared in 10 p.c. horse serum saline and inoculated into the allantoic cavity of 11-day chick embryos. After 2 days' incubation at 35° C. the eggs were examined for the presence of haemagglutinin and positive eggs inoculated with the limit infecting dilution (at which it may be assumed that virus develops from a single infective unit) and the dilution below this were harvested and further examined.

Marker tests. In earlier work on recombination between MEL and WSE, virus fluids obtained by these means were submitted to a series of six tests each of which clearly distinguished MEL from WSE. Using these marker tests it was possible to establish the presence in these fluids not only of the parent strains but of the two recombinant types M+ and WS— each of which combined characters derived from both the parents. In investigating the yield of recombinant types using M+ and WS— as the starting strains, the same series of marker tests was used. Using the capital letter to denote the MEL character and the small letter for the WSE character the marker tests are as follows.

Aa. Serological type. This was determined by haemagglutinin inhibition using hyperimmune rabbit sera prepared against MEL and WSE. Serial dilutions of serum were prepared in 0·25 ml. volumes in normal saline and to each were added 5 agglutinating doses (A.D.) of virus followed by 0·25 ml. 1 p.c. fowl red cells. The end-point was represented by the tube showing the standard pattern of partial agglutination after the cells had settled for 45 minutes at room temperature.

Bb. Heat resistance. MEL haemagglutinin is resistant to heating at 60° C. for 30 minutes whereas WSE haemagglutinin is destroyed under these conditions.

Cc. Conversion to indicator. Heating of the virus fluid to 52·5° C. for 30 minutes results in a change to the indicator state in the case of WSE, i.e. the haemagglutinin can then be inhibited by small amounts of soluble mucoid such as ovomucin or meconium. MEL, on the other hand, is not converted to indicator under these conditions.

Five agglutinating doses of heated virus are added to serial tenfold dilutions of mucoid inhibitor in 0·25 ml. volumes in normal saline. After allowing 30 minutes at room temperature for virus-inhibitor union to take place, 0·25 ml. 1 p.c. fowl red cells are added and the end-point determined 45 minutes later from the pattern of agglutinated cells.

Dd. Inhibition of indicator virus haemagglutinin by sheep salivary mucoid. When WSE is converted to indicator by heating at 52·5° C., the haemagglutinin is strongly inhibited by high dilutions of sheep salivary mucoid (McCrea, 1951). No treatment of MEL has ever yielded haemagglutinin inhibited by this substance.

Ee. Production of haemorrhagic lesions in the chick embryo. The inoculation of 0·05 ml. of a 10^{-2} dilution of WSE on to the chorioallantoic membrane of 12-day chick embryos usually results on the third or fourth day after inoculation in death of the embryo with haemorrhagic encephalitis and multiple haemorrhages in the muscles and lungs. Embryos inoculated similarly with MEL are usually alive after 4 days' incubation and show no macroscopic abnormalities.

Ff. Pathogenicity for mice by the intranasal route. Intranasal inoculation of 0·2 agglutinating doses of WSE into 4-week-old mice results in death of the mouse within 3–5 days

with complete pulmonary consolidation. Similar inocula of MEL produce non-fatal lesions seldom involving more than a quarter of the total lung area.

In terms of these marker characteristics the four virus types may be represented thus:

MEL	M+	WS—	WSE
ABCDEF	ABcDeF	abCdEf	abcdef

EXPERIMENTAL.

First experiment on recombination between M+ and WS—.

This experiment was along standard lines, a mixture of freshly harvested allantoic fluids, one part WS— (haemagglutinin titre (HA) = 350) to two parts of M+ (HA = 200) being used to replace 4 ml. of allantoic fluid. In each of 4, 11 day eggs two eggs were similarly treated with WS— alone and two with M+ alone. The fluids were harvested 7 hours after infection and gave the findings shown in Table 1.

TABLE 1.

Character of first cycle fluids.

Egg No.	Inoculum	HA	Serology Anti MEL	Serology Anti WS	Infectivity titration ID_{50}
87457–60	M+/WS	40	<200	350	$10^{4.8}$
87461–62	WS—	25	<200	600	$10^{3.0}$
87463–64	M+	30	2400	<100	$10^{4.1}$

HA. haemagglutinin titre.

Serology. Dilution of anti-MEL or anti WS ferret serum inhibiting haemagglutination by 5 ADs. of virus fluid.

Infectivity titration—ID_{50} per 0·05 ml. of virus fluid determined by allantoic inoculation of eggs.

Several fluids at limit infective dilution (LD fluids) from titration of control fluids 87461–2 (WS—) and 87463–4 (M+) were shown to be of the type expected and are not further considered.

Allantoic titration of the first cycle fluid pool 87457–60 WS—/M+ gave LD fluids whose *in vitro* character is shown in Table 2.

TABLE 2.

Types of fluid obtained by LD titration of first cycle fluid.

Dilution	M+	WS—	MEL	WSE	Negative	Not tested
$10^{5.5}$	1				8	
$10^{5.0}$	7	7			19	2
$10^{4.5}$	14	7	3	1	5	6
Total	22	14	3	1		

In this experiment it will be noticed first that a high proportion of the yield is of incomplete virus. Based on Fazekas and Cairns' 1952 findings, a haemagglutinin titre of approximately 30 corresponds to an ID_{50} of $10^{7 \cdot 1}$ if all virus is complete. The values obtained indicate that only 1 : 200 to 1 : 10,000 of the haemagglutinating particles were infective. Only a small yield of apparent recombinants was obtained. A third point of interest is that the primary fluid from the mixed infection showed an excess of haemagglutinin neutralizable by anti WS serum, but that amongst the infective fluids isolated there is a clear predominance of M+ and MEL as against WS types.

The four apparent recombinants were studied in detail.

Behaviour of MEL type primary fluids 87699, 87709, 87610.

Fluid 87699 showed typical MEL findings except that after heating to 52·5° C. for 30 minutes the haemagglutinin was partially inhibited by 10^{-2} dilution of meconium, the lowest dilution tested. On titration, however, it gave 1/8 positive fluids at 10^{-8} 3/4 at 10^{-7}, all of which were typical M + *in vitro;* one tested *in vivo* gave typical M+ reactions.

TABLE 3.

Characterization of apparent recombinant strains in vitro.

Origin	Fluid tested 1st LD	Fluid tested 2nd LD	*In vitro* character	C.A. test	Mouse intranasal	Constitution
87699	87814		M+	4–0/4	0·9	ABcDeF
87709	87830		MEL	0–3/4	1·8	ABCDEF
		87945	MEL	0–0/3	0·5	ABCDEF
		87948	MEL	0–2/3	2·2	ABCDEF
87610			MEL	3–0/3		ABCDe
	87782		MEL	1–3/4	1·9	ABCD(e)F
		87965	MEL	3–0/3	1·1	ABCDeF
		87967	MEL	0–1/3	0·5	ABCDEF

C.A. test. Virus diluted 1: 100 inoculated on chorioallantois of 3 or 4 12-day embryos. Results, 4–0/4 = four embryos dead with haemorrhagic lesions, none dead without lesions out of 4 inoculated. 0–1/3 = one embryo dead without haemorrhages of three inoculated, the other two alive.

Mouse intranasal. Average lung lesions of 4 mice inoculated intranasally with 1 AD of virus fluid. 5 = death by 5th day; 4, death with complete consolidation on 6th or 7th day, 3, 2, 1 diminishing degrees of consolidation.

Fluid 87709 gave consistent MEL reactions through two further limit dilution titrations. First passage gave 1/8 positive at 10^{-8} (87830), this fluid on titration gave 1/4 positive at 10^{-9} (87945) and 6/6 at 10^{-8} including fluid 87948. *In vivo* tests of these fluids are shown in Table 3. All have the constitution ABCDEF. Fluid 87610 gave MEL reactions *in vitro,* but when tested on the chorioallantois killed embryos with specific haemorrhagic lesions. Lungs from two of these contained enough virus for presumptive *in vitro* test; one

showed MEL one M+. Allantoic titration of 87610 gave 1/8 positive (87782 *in vitro* MEL) at 10^{-8}, 3/4 at 10^{-7} 2 MEL 1 M+. Titration of 87782 gave 5/6 all MEL (87964–5–7) except 87966 which had M+ character. Titration of this M+ fluid gave no positive fluids at 10^{-8} and 7/9 positive at 10^{-7}. These showed one with typical inhibition by meconium, the others showed varying degrees of partial inhibition, making it difficult to decide whether the type should be given as MEL or M+. The *in vivo* characters of these fluids are shown in Table 3.

In summary one typical MEL recombinant was obtained; another fluid gave a series of descendant forms which retained the capacity to give a variety of intermediate findings including the complex ABCDeF. We are well aware that the results can be interpreted simply as a failure to obtain pure clone fluids and that for 3 successive generations mixed MEL and M+ fluids were obtained at limiting dilutions. This type of result is referred to in the Discussion.

Behaviour of fluid 87606 (WSE). This fluid tested at once on the chorio-allantois killed embryos without haemorrhagic lesions. Limit dilution titration allantoically in eggs gave 3/8 positive at 10^{-8} M+ 87766 and 2 WSE 87768–9 with abnormally high neutralization by anti MEL serum. The 4/4 fluids at 10^{-7} included one typical M+ and 3 resembling WSE but also showing significant neutralization by anti MEL serum.

Two of the WSE fluids were titrated, giving consistent WSE findings *in vitro* with normal serological reactions. There were, however, interesting variations in the reactions *in vivo*.

TABLE 4.

Characterization in vivo *of WSE derivatives.*

Orig.	1st LD	2nd LD	*In vitro* type	C.A. test	Mouse intranasal	Constitution
87606			WSE	0–3/3		abcdE
	87766		M+	2–2/4	3·1	ABCDe(f)
	87768		WSE	0–4/4	4·5	abcdEf
		87939	WSE	0–3/3	5·0	abcdEf
		87940	WSE	0–3/3	4·5	abcdEf
		87941	WSE	1–2/3	5·0	abcd(E)f
		87942	WSE	0–3/3	4·5	abcdEf
	87769		WSE	0–4/4	2·8	abcdEF
		88024	WSE	1–2/3	4·3	abcd(E)f
		88031	WSE	0–3/3	1·7	abcdEF
		88033	WSE	2–1/3	4·5	abcdef
		88035	WSE	0–3/3	2·7	abcdEF

Symbols as Table 3. Where a symbol is bracketed under constitution the result is not fully typical, e.g. (f) indicates that lesions averaging more than 3 were observed in mice which is more than those normally found with MEL, but less than the result >4 characteristic of WS strains.

Another series of passages was initiated by titrating 87606 in the presence of anti MEL serum 1 : 100. Five limit dilution fluids were characterized and

each again re-isolated and characterized. All were WSE *in vitro* while the *in vivo* characters Ee and Ff were as shown.

1st LDs	Ef	(e)f	Ef	ef	(e)f
2nd LDs	Ef,(E)f	ef,ef	Ef,Ef	(e)f,Ef	(e)f,(e)f

In summary this fluid in addition to providing one atypical M+ has given rise to WSE forms with three distinct patterns of pathogenicity abcdef, abcdEf and abcdEF as well as some indeterminate forms.

Second recombination experiment.

This experiment differed from that already described in that the allantoic cavities were washed once with Ringer solution before the virus was inoculated and a lower concentration of each virus was used. Each virus was used at a dilution corresponding to a H.A. titre of 20. Otherwise the technique followed the standard procedure, in addition to the M+/WS— mixture other eggs were inoculated with M+ and WS— alone.

The first cycle fluids from eggs inoculated with these latter single strains were all of normal character, LD fluids from titration *in ovo* gave 11 M+ fluids and 8 WS— fluids all with the expected *in vitro* qualities. One representative of each had typical characters *in vivo* and a further LD titration from these showed no anomalous fluids.

The pooled first cycle fluid from 2 eggs inoculated with M+/WS— mixtures had an HA titre of 140 and showed what we refer to as anomalous neutralization, both anti MEL and anti WS sera showing significant inhibition of haemagglutination.

TABLE 5.

Titration of first cycle fluid (92152–5) from M+/WS— inoculum.

	1st and 2nd titrations						3rd titration with anti MEL serum			
Dilution	HA+	Types					HA+	Types		
10^{-8}	0/11									
10^{-7}	4/20	2WS—	1M+		1+					
10^{-6}	16/27	4WS—	6M+		6M		12/20	10WS—	1M+	1WSE
$10^{-5\cdot5}$	16/17	7WS—	2M+	4M	1WSE	2+	12/20	5WS—	4M	3WSE
10^{-5}	26/27	9WS—	13M		1WSE	3+				

HA+ proportion of eggs showing development of haemagglutinin.
Under Types, + = positive fluid not further characterized or giving anomalous result.
M = MEL.

Three separate limit dilution titrations of the first cycle fluid pool 92152–55 (M+ and WS—) were made, two direct and one in which the diluent was anti MEL serum 1:25 (Table 5). There are several interesting features in these results. If we take the determinations *in vitro* at their face value, the

approximate ID_{50}s for the four types are WS— 6·1, M+ 5·5, MEL 5·5, WSE 5·0. In the results at a dilution of $10^{5.0}$ there is clearly an excess of MEL and, as in previous experiments, we can be sure that in most instances when MEL and another type are simultaneously inoculated MEL will predominate and give the *in vitro* character of the fluid obtained.

The primary objective of this experiment was to show that from the recombinants WS— and M+ the original forms MEL and WSE could be obtained by back recombination. Attention was therefore concentrated on those fluids which had MEL or WSE character or showed equivocal reactions. Only two samples each of M+ and WS— fluids were further studied. These after re-isolation from limit dilution gave the typical *in vitro* and *in vivo* reactions of their type. The results with further limit passage of the primary fluids shown in Table 5 as of MEL or WSE character are given in the following sections. Study of two anomalous fluids was also undertaken with very complex results which in the interests of brevity will not be reported.

Primary fluids of MEL character.

Four fluids 92527, —528, —532, 92936 taken through two series of limit dilution passages and fully tested, were consistently MEL in character. Two others, 92525 and 92531, gave one fluid of other character in the first limit dilution titration, but on the second gave all typical fluids. There were some differences in the activity *in vivo* amongst the strains.

TABLE 6.

In vivo *characters of MEL fluids.*

Primary Fluid	C.A. test		Mouse intranasal	
	1st LD	2nd LD	1st LD	2nd LD
92525	0–3/7	0–0/4	0·7	1·6
527	0–0/3	0–0/4	0·7	0·5
528	0–3/7	0–1/4	2·1	2·2
531	0–2/4	0–2/4	1·0	0·2
532	0–3/4	0–4/4	0·8	1·9
92936	0–2/4		0·9	

Symbols as Table 3. One representative fluid, MEL *in vitro* from each limit titration shown.

It will be seen from Table 6 that 92528 is significantly more pathogenic for the mouse lung and 92532 significantly more lethal for chick embryos although in neither instance does the increased pathogenicity approach the level characteristic of WSE.

Three fluids require individual consideration: Fluid 92518 from 10^{-6} dilution of the first cycle fluid gave all the *in vitro* reactions of MEL, but on titration 1 WSE and 4 MEL fluids were obtained from the limiting infective dilutions. The WSE fluid 92611 on further limit dilution passage gave WSE

with pathogenicity for both chick embryos and mice significantly less than standard (1–3/4, 3·9). One of the MEL fluids (92614) gave WSE type reactions in both chick embryos and mice and on limit dilution gave at 10^{-9} two fluids one 92885 (with WS serology), which was of such low titre that it could not be further characterized and the other 92886 with all MEL characters except for a slightly increased susceptibility to inactivation by anti-WS serum. One further passage gave completely typical MEL fluids. A retitration of 92518 gave all MEL fluids at limit dilution, but one of these when tested on the chorioallantois gave 1–2/4 and from the haemorrhagic lesions a virus with WS serology was obtained.

These results suggest strongly that in 92518 the dominant component has MEL somatic character but is in some way associated with the capacity to give rise to WS type particles on passage. The possibility of a simple mixture cannot be wholly excluded, however.

Fluid 92530 gave typical findings for MEL *in vitro* but on titration gave 10^{-9} M+ WS—, —, —, 10^{-8} all MEL. A repetition showed that the titre had fallen, but at 10^{-7} 2 MEL and one M+ fluids were obtained.

In summary at least 6 indubitable MEL fluids were obtained as recombinants of M+/WS—. In addition, two fluids were obtained which with some regularity gave on limit dilution types other than that conferring the *in vitro* character on the fluid.

Primary fluids of WSE character.

Three fluids 92947, 93222 and 93250 gave no anomalous results on two successive limit dilution titrations. Similarly the fluid 92611 derived from the MEL fluid 92518 above gave typical findings, apart from somewhat less than standard pathogenicity. Fluid 93256 had the characteristics of WSE except that it was neutralized by anti MEL serum to a titre of 400 as well as being normally neutralized to a titre of 600 by anti WS serum. Titration to limit dilutions gave 3 WSE fluids, one of which 93338 had typical reactions *in vivo* and in turn gave typical WSE fluids on further limit passage. Fluid 93253 (typical WSE *in vitro*) on limit dilution passage gave 3 fluids with WS— characters. These were not further studied.

TABLE 7.

In vivo *characters of WSE type fluids.*

Primary fluid No.	C.A. test		Mouse intranasal		Constitution
	1st LD	2nd LD	1st LD	2nd LD	
(92518 MEL)	1–2/3	3–1/4	3·9	3·9	e (f)
92947	4–0/4	4–0/4	4·0	3·7	e (f)
93222	3–1/4	2–2/4	3·2		e (f)
93250	2–2/4	4–0/4	4·75		e f
93256	4–0/4	4–0/4	5·0		e f

In summary, 2 typical and 3 slightly atypical WSE fluids have been obtained in this experiment. All have the *in vitro* reactions of the type with the *in vivo* reactions shown in the Table.

DISCUSSION.

The primary object of these experiments was to show whether or not the reciprocal recombinants obtained in previous experiments from mixed infections of MEL and WSE, could give rise to the original forms in back-recombination tests. Both experiments gave unequivocally positive results but, in addition, provided a rich crop of anomalies which may prove to have significance for the interpretation of influenza virus genetics.

It is first necessary to mention the possibility of contamination playing a part in the results. Every care was taken but with dozens of virus fluids being characterized each day with *in vitro* tests, the possibility of an occasional contaminant entering an egg at the time of inoculation can hardly be excluded. To get some estimate of its likelihood a search was made through the records of titrations made during the currency of these experments. A total of 672 eggs inoculated with dilutions beyond the infective level remained negative on both 2nd and 3rd day tests. In 3 instances only a dilution showed positive findings when the dilution below it gave all negative results. In 2 of these instances the virus obtained was of the "correct" type, in one it was of an unexpected type. This is the only instance in the present work where we feel reasonably certain that contamination occurred and the finding was discarded.

Otherwise all the results obtained in the series selected for report have been fully stated and the results will be accepted at their face value in the rest of this discussion.

In the first experiment there was, as would be expected, a much higher proportion of non-infective virus than in the second in which the inoculum was much smaller (approximately 1 virus particle of each type per cell). In the second experiment, too, there was a higher yield of recombinants.

Before discussing some of the anomalous strains obtained, it should be stated that the stock strains MEL and WSE have proved completely consistent in all the tests used for their characterization. So too, have the strains of WS— and M+ used in the present work including all the fluids tested from first cycle fluids from eggs infected with either M+ or WS— alone. The anomalies described must therefore be due (a) to recombination or some equivalent type of genetic interaction; (b) to the production of heterozygotes in the sense of morphological units carrying two or more different genomes or (c) to the existence of unresolved mixtures.

The evidence has been given to establish the recovery of a relatively large number of strains of MEL character and at least 2 of WSE character which

bred true on repeated limit titration. These provide the evidence for back recombination which was the primary objective of the work.

Many anomalous results have been encountered, by no means all of which are recorded in this paper. Taken along with other lines of evidence to be presented in future communications especially the phenomenon of "anomalous neutralization" mentioned above in relation to the first cycle fluid from the second experiment, our impression is that heterozygote production undoubtedly occurs and as a result gives mixed clones which are very difficult to interpret. Many of the anomalous fluids noted in the Tables may well represent essentially pure clones of the genetic constitution indicated. If, however, we admit the possibility of heterozygote formation followed by various types of dissociation and recombination it becomes almost impossible to provide a rigid proof of this. It will probably be necessary to seek a simpler system involving single known mutational steps between the components before effective work in this field is possible.

A point of interest is the distribution of the four *in vitro* types amongst the primary fluids obtained from first cycle fluids in experiments of the present type. We may tabulate the results (1) from the MEL/WSE experiment described by Burnet and Lind, 1952, (2) from Experiment 2 of the present paper and (3) from a similar experiment as yet unpublished carried out by the de-embryonated egg technique, in Table 9.

TABLE 9.

Distribution of in vitro *types in first cycle fluids.*

	MEL	WS—	M+	WSE
1. MEL/WSE	22	9	6	4
2. M+/WS—	23	22	9	2
3. M+/WS—	13	9	6	1

In other experiments there have been excess numbers of the two initial viruses but there is a clear general tendency for a similar distribution of strains to be produced whichever way the recombination experiment is carried out. The order of abundance is MEL$\geqslant$WS—$>$M+$>$WSE. There is evidence from some experiments that the distribution of incomplete (non-infective but haemagglutinating) virus may differ from that found for infective virus by the present technique. We feel that this characteristic distribution of the four types may well be a manifestation in part of the formation of a proportion of multiple-genome units. Further development of this theme must wait for the accumulation of more experimental material.

SUMMARY.

The strains M+ and WS— obtained as recombinants from MEL and WSE strains of influenza A virus in previously reported experiments, have been used in back-recombination experiments to produce MEL and WSE.

Several strains of each type have been obtained as well as a number of anomalous strains whose nature is discussed in the text.

Irrespective of the way the experiment is carried out, MEL plus WSE or M+ plus WS—, the order of abundance of the four products in technically satisfactory experiments is MEL WS— M+ WSE.

Preliminary evidence for the existence of "heterozygotes" is presented.

Acknowledgment. We wish to acknowledge the highly competent technical assistance of Lois Larkin during the course of this work.

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INDOLE AND SKATOLE IN THE MILK OF THE RUMINANT FEEDING ON *LEPIDIUM* SPP.

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Practical aspects of the weed taint problem in Australia have been dealt with in an earlier paper (Conochie, 1950). Various species of the genus *Lepidium* (chiefly *L. hyssopifolium* and *L. bonariense*) at times provide the greater part of the herbage available to dairy stock in some districts of southern Queensland. The milk produced has a faecal odour and a persistent nauseating flavour due to the presence of abnormal amounts of indole and skatole. Hussong and Quam (1943) found that butter from the milk of cows feeding on *Lepidium virginicum* had a high indole content, but they were not able to produce the effect with animals feeding under controlled conditions, nor did they investigate the physiological processes involved. Combs (1949) reported that the effect had been produced under controlled conditions in 1935–37 with both *L. virginicum* and a species of the genus *Ambrosia*.

There is a complete absence in the literature of basic information on the production, absorption and excretion of indole and skatole in the normal ruminant. In addition nothing is known of the effect of *Lepidium* in the diet on these processes. A study of the subject was undertaken in the hope that it might suggest some practical steps, such as the control of feeding practice, which would reduce the incidence of *Lepidium* taint under field conditions.

Lepidium hyssopifolium, the most widespread species of the genus in Australia, was used in all tests.

METHODS.

Milk indole and skatole. The method used for the estimation of milk indole was based on that of Clarke *et al.* (1937). The p-dimethylaminobenzaldehyde complex was measured in an Evelyn photoelectric colorimeter with filter No. 540. Since skatole also forms a coloured complex with appreciable absorption at 540 mμ, results obtained by the Clarke method will reflect both the indole and skatole contents. As shown by the results quoted later, both substances are present in *Lepidium*-tainted milk. However, results throughout this report are expressed as indole, calculated from the total absorption.

Separate values for indole and skatole were obtained in a few cases by measuring the absorption of the p-dimethylaminobenzaldehyde complex at 540 mμ and 570 mμ in a Beckman spectrophotometer. The absorption curves for the pure complexes of indole and skatole with p-dimethylaminobenzaldehyde are shown in Fig. 1.

Since this work was completed, Myers (1950) has published a method for the co-determination of indole and skatole by the use of a modified p-dimethylaminobenzaldehyde reagent.

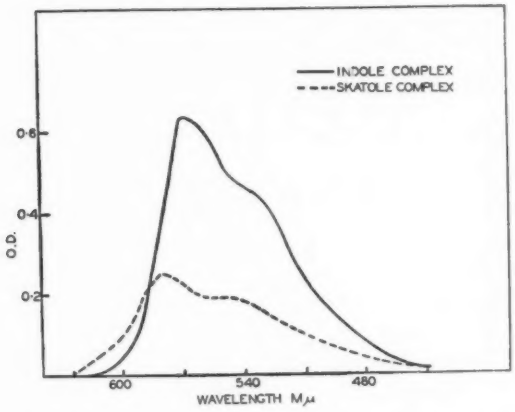

Fig. 1. The absorption curves for the complexes of indole and skatole with p-dimethylaminobenzaldehyde.

Urinary indican. The method of Sharlit (1934) as modified by Meiklejohn and Cohen (1942) was used. It is based on the formation of a coloured complex of indoxyl with thymol. According to Sharlit (1934) the presence of potassium persulphate prevents interference from skatole. Tests made showed that the result was almost unaffected by free indole in amount equivalent to the amount of indoxyl. The final colour measurement was made in an Evelyn photoelectric colorimeter with filter No. 540.

Faecal indole. An aliquot of 100 ml. of the faecal macerate was washed into a Quickfit distillation flask with 200 ml. distilled water. Paraffin wax was added to prevent frothing and 500 ml. of distillate were obtained leaving about 100 ml. of residue in the distillation flask. An aliquot of the distillate was acidified with HCl and analysed for indole by the method of Happold and Hoyle (1934): an Evelyn photoelectric colorimeter (Filter No. 540) was used for the colour measurement.

Blood indole was estimated by the method of Macchia (1935).

RESULTS.

The indole and skatole content of normal milk. No special study has been made of the indole and skatole content of normal milk, but the results presented in this paper suggest that these compounds are normally present in the milk fat of the cow or goat in concentrations ranging from 0·05 to about 0·3 p.p.m.

Precursors of indole and skatole. The first possible source of indole and skatole in tainted milk to which attention was directed was the occurrence in the plant of the compounds themselves or of precursors requiring only slight modification in the alimentary tract. Steam distillation of *L. hyssopifolium* gave only traces of a substance forming a coloured complex with p-dimethylaminobenzaldehyde. The amount was too small to account for the indole and skatole appearing in the milk.

As indole and skatole are generally considered to be produced in the large intestine from tryptophane, estimations were made of the amount of this amino acid in the plant protein. Assay by the chemical method of Lugg (1937) and

by a microbiological method[1] established that *Lepidium* had a tryptophane content of 1·6 gm. per 16 gm. N, which is normal according to Lugg (1941). The protein content of *Lepidium* ranged from 27 to 32 p.c. in the dry matter. If a high tryptophane content was responsible it would be expected that rations of high quality protein would also result in high indole and skatole levels in milk. No such effect has been observed.

Production of indole in the rumen. In the absence of an abnormal amount of a known precursor of indole and skatole, attention was given to the effect of the plant on indole production in the rumen. Pearson and Smith (1943) found that *in vitro* incubation tests could be carried out at 39° C. with strained rumen contents, using a slow stream of CO_2, provided the period of incubation did not exceed four hours: under these conditions there was little change in pH or in the composition of the micro-flora.

This technique was used to investigate the effect of macerated *Lepidium* on indole production, using rumen contents from sheep. The incubation time was 4 hours. Indole was estimated, by the method of Happold and Hoyle (1934), in steam distillates obtained from the macerates made alkaline with KOH.

The results given in Table 1 show that indole production in ruminal fluid was not greatly affected by the presence of *Lepidium*.

TABLE 1.

Effect of Lepidium *on the production* in vitro *of indole from rumen contents.*

		Indole (p.p.m. in fluid)
Sheep 1	Fluid alone 250 ml.	0·52
Sheep 1	Fluid + *Lepidium* 20 gm.	0·62
Sheep 2	Fluid alone	0·43
Sheep 2	Fluid + *Lepidium* 20 gm.	0·39

The rôle of micro-organisms in indole production. If the indole and skatole which appear in the milk are produced by coliform organisms in the alimentary tract, the feeding of antibiotics could be expected to influence the amount produced. An experiment was conducted in which phthalylsulphathiazole was fed to a goat (45 lb. body wt.) every four hours from 9 a.m. to 9 p.m. at the rate of 5, 8, 8, 11, 12, 12, 12, and 9 gm. per day on eight successive days. (Quarmby, 1949, reports that 1 gm. sulphathiazole per 20 lb. body wt. is effective in controlling entero-toxaemia in goats.) After 5 days, coliform organisms were absent from the faeces, as determined by the use of MacConkey's plates, but spreading films appeared on the anaerobic blood agar plates. Portion of the lucerne ration was then replaced with *Lepidium* at the rate of 2 lb. of leaves per day, and administration of the drug was continued. Within 24 hours of feed-

[1] Acknowledgment is due to Miss J. Millis and Dr. Nancy Hayward for the microbiological assay.

ing the goat on *Lepidium*, the indole content of the milk had increased sixfold while coliform organisms remained absent from the faeces. The anaerobic organisms giving a spreading film on the blood agar plate remained throughout the test.[2] Two and a half days after the drug was discontinued coliform organisms reappeared in the faeces, and the indole content of the milk fat rose to about ten times the normal value. The results are given in Fig. 2.

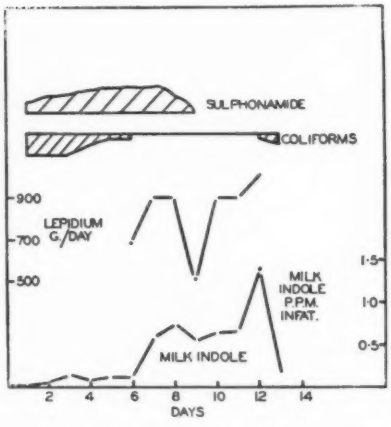

Fig. 2. Showing the influence of phthalylsulphathiazole, given by mouth, on the indole content of the milk and on the abundance of coliform organisms in the faeces of goat No. 1 feeding on a *Lepidium*-containing diet

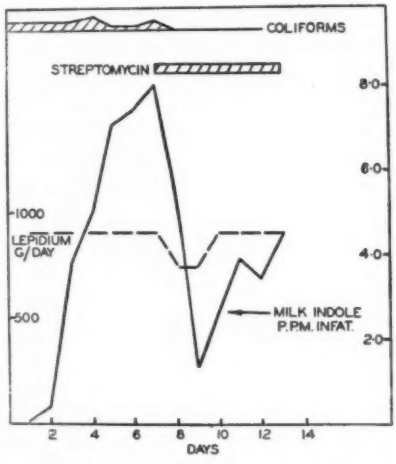

Fig. 3. Showing the influence of streptomycin, given by mouth, on the indole content of the milk and on the abundance of coliform organisms in the faeces of goat No. 1 feeding on a *Lepidium*-containing diet.

In view of the failure of phthalylsulphathiazole wholly to prevent tainting by *Lepidium*, a further test was made with dihydrostreptomycin. Whereas in the previous test the drug was given before the *Lepidium*, in this test *Lepidium* was given until the indole content of the milk had reached a high level and then the drug was administered. Coliform organisms were eliminated from the faeces by the streptomycin within 24 hours, but the numbers of spreading colonies growing on both aerobic and anaerobic blood agar plates remained unchanged throughout the test. The results given in Fig. 3 show that when streptomycin was administered the indole content of the milk dropped sharply but rose again to a fairly high level. The sharp fall in indole content of the milk may have been partly due to a reduction in food intake for two days when the streptomycin was first given.

[2] Dr. Nancy Hayward and Mr. H. N. Paine carried out the bacteriological analyses of the faeces.

Tryptophanase activity. From incubation of macerated *Lepidium* with bacto-tryptone no evidence for the presence of a plant tryptophanase could be obtained. In addition, a buffered extract of the plant when incubated with a tryptophanase preparation from *Esch. coli* was found to inhibit the enzyme to a greater extent than did a lucerne extract.

The influence of Lepidium *on the excretion of indole.* Indole which is absorbed from the large intestine is oxidized chiefly in the liver (Nicolai, 1942) to indoxyl and this is then converted to either the ethereal sulphate or the glucuronide which is excreted in the urine. Skatole is believed to be dealt with in a similar manner. In man the greater proportion of absorbed indole is excreted as ethereal sulphate (Tollens, 1910; Novello *et al.*, 1925). It is shown elsewhere in this paper that more than three-quarters of a quantity of indole given by mouth to a goat was excreted as ethereal sulphate.

To determine the influence of *Lepidium* in the diet on the excretion of indican, and on the indole content of the milk, experiments were conducted with a milking goat, held in a metabolism crate.

TABLE 2.

The daily excretion of urinary indican and the indole content of milk.

(Goat No. 1)

On Lucerne			*On Lepidium* (815 gm./day)		
	Indican (Expressed as indole gm./day)	Milk indole (p.p.m. in fat)		Indican (Expressed as indole gm./day)	Milk indole (p.p.m. in fat)
1949			1949		
16/8	0.24	0.40	30/7	0.20	0.95*
17/8	0.13	0.10	31/7	0.17	0.55*
18/8		0.10	1/8	0.35	0.95
19/8		0.10	2/8	0.22	1.30
20/8	0.07	0.05*	3/8	0.31	4.75
21/8	0.17	0.05*	4/8	0.41	2.90
22/8	0.26	0.30	5/8	0.34	9.20
23/8	0.29	0.15	6/8	0.22	2.55*
24/8	0.26	0.80	7/8	0.23	0.95*
25/8	0.32	0.15	8/8	0.54	2.70
26/8		0.25	9/8	0.21	3.80
27/8	0.25	0.10*	10/8	0.19	5.60
28/8	0.28	0.10*	11/8	0.23	4.80
29/8	0.31	0.15	12/8	0.30	5.10
30/8		0.40	13/8	0.10	0.95*
31/8	0.13	0.10	14/8	0.24	1.35*
1/9		0.35	Av.		
2/9		0.40	(16 days)	0.27	
3/9	0.29	0.15			
4/9	0.27	0.10*			
5/9	0.25	0.15*			
Av. (15 days)	0.24				

* once a day milking.

TABLE 3.

Daily excretion of urinary indican and faecal indole and the indole content of the milk.

(Goat No. 2)

On Lucerne				*On Lepidium*			
Date 1950	Indican (expressed as indole gm./day)	Faecal indole (Mg.)	Milk indole (p.p.m. in fat)	Date 1950	Indican (expressed as indole gm./day)	Faecal indole (Mg.)	Milk indole (p.p.m. in fat)
31/10	0.37		0·04	28/11	0.44	7.5	1·78
1/11	0.47			29/11	0.48	9.8	
2/11	0.39		0·16	30/11	0.42	9.8	1·20
3/11	0.42			1/12	0.43	7.1	1·52
4/11	0.58		0·09	2/12	0.46	7.4	2·2
5/11	0.47			3/12	0.51	9.5	0·59
6/11	0.48			4/12	0.51	8.5	
7/11	0.47		0·31	5/12	0.35	6.3	6·42
8/11	0.56			12/12	0.31	7.0	1·0
9/11	0.37	5.7		13/12	0.33	4.0	
10/11	—			14/12	0.40	3.9	
11/11	0.43	6.5	0·23	15/12	0.34	2.7	1·7
12/11	0.46						
13/11	0.42	3.9	0·70				
14/11	0.41	4.0					
15/11	0.39	5.8					
6/12		4.5	0·10				
7/12		3.0					
8/12		11.2					
9/12		5.2					
10/12		4.9					
11/12		3.9					
Average	0.45 gm.	5.3 mg.		Average	0.42 gm.	7.0 mg.	

The urine was collected into an equal volume of N/10 HCl, measured and analysed for indican. Faeces were collected into a 0·4 p.c. solution of KOH which was constantly stirred to ensure rapid maceration of the faecal pellets. The pH of the macerate was about 10. Indole was estimated on an aliquot of the macerate.

The ration consisted of a mixture of fresh lucerne and dry cereal chaff. When *Lepidium* was given it replaced portion of the green lucerne. Only leaves and succulent shoots of *Lepidium* were used, any woody material being discarded.

The results of three tests are given in Tables 2, 3 and 4.

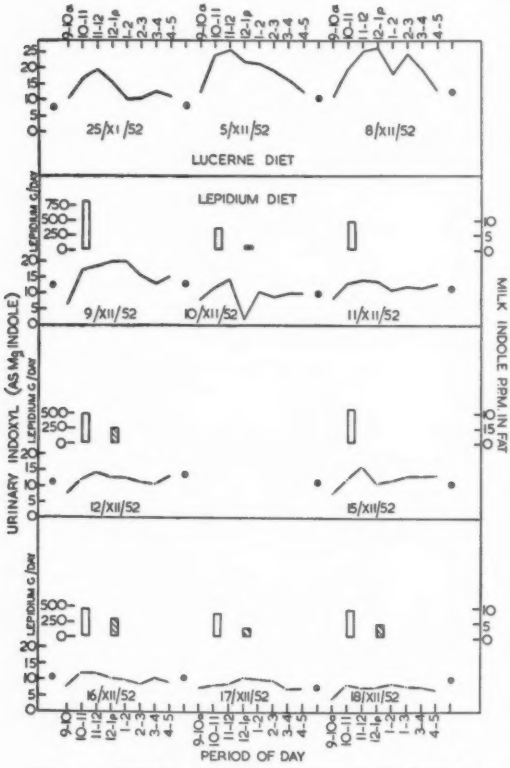

Fig. 4. Showing the hourly excretion of urinary indoxyl (expressed as mg. indole) by goat No. 3 feeding on lucerne and *Lepidium*-containing diets.

O indicates average hourly overnight excretion.

Shaded panel indicates milk indole levels.

Open panel indicates *Lepidium* consumed.

From these results it is evident that the amount of indole detoxicated and excreted daily is not greatly affected by the inclusion of *Lepidium* in the diet. The indole content of the milk was greatly affected by feeding *Lepidium*, and this was also evident from the odour of the milk.

The influence of Lepidium *on the rate of excretion of urinary indican.* An attempt was made to measure the hourly rate of excretion of urinary indican by passing a self-retaining catheter into the bladder of a female goat. The small diameter of the urethra so limited the size of the catheter which could be used, that blockages were frequent. In addition evidence was obtained that even when the catheter flowed freely it did not always empty the bladder completely. It was found later that by spraying the animal's feet with water it could be made to urinate hourly. By this technique hourly samples were collected

between 9 a.m. and 5 p.m. and the overnight urine was collected in the usual way from the metabolism crate, urination being initiated at 9 a.m. by the hosing technique to ensure a complete sample.

Tests were made with a goat on both lucerne and *Lepidium* diets. The results are presented in the graphs of Fig. 4.

It is apparent from these results that when the animal was feeding on a normal diet the rate of indican excretion rose to a peak of over 25 mg. per hour (expressed as indole) about mid-day. When the diet contained *Lepidium* although the milk contained free indole the rate of excretion remained at a level of less than 15 mg. per hour except on the first day after the changeover from lucerne to *Lepidium*.

TABLE 4.

The daily excretion of urinary indican.

(Goat No. 3)

On Lucerne		*On Lepidium*	
Date 1952	Indican (expressed as indole gm./day)	Date 1952	Indican (expressed as indole gm./day)
23/11	0.27	9/12	0.32
24/11	0.28	10/12	0.24
25/11	0.22	11/12	0.27
5/12	0.31	12/12	0.31
8/12	0.35	15/12	0.26
		16/12	0.25
		17/12	0.18
Av. (5 days)	0.29	Av. (7 days)	0.26

TABLE 5.

The excretion of urinary indican during the day and night.

On Lucerne				*On Lepidium*			
	Urinary indican (expressed as indole) mg.				Urinary indican (expressed as indole) mg.		
Date	9 a.m.–5 p.m.	5 p.m.–9 a.m.	Total	Date	9 a.m.–5 p.m.	5 p.m.–9 a.m.	Total
23/11	136	130	266	9/12	122	199	321
24/11	158	117	275	10/12	84	154	238
25/11	102	120	222	11/12	92	177	269
5/12	146	163	309	12/12	92	218	310
8/12	152	194	346	15/12	95	169	264
				16/12	78	170	248
				17/12	66	113	179
Average	139	145	284	Average	90	171	261
(5 days)	49 p.c.	51 p.c.	100 p.c.	(7 days)	34.5 p.c.	65.5 p.c.	100 p.c.

The results presented in Table 5 show that with a lucerne diet, the animal excreted 49 p.c. of its daily output of indican during the 8-hour day, whereas with a *Lepidium*-containing diet only 34·5 p.c. of the indican output was produced during the 8-hour day. It would appear therefore that *Lepidium* interferes in some way with the normal detoxication of indole.

The influence of Lepidium *on the* in vivo *production and absorption of indole.* The faecal indole and urinary indican figures given in Table 3 suggest that *Lepidium* has little influence on the overall production of indole. It is further evident from these figures that with the goat a high proportion (99 p.c.) of the indole produced in the intestine is absorbed. The results, set out in Table 6, of a test in which indole was fed to a goat, feeding on lucerne, at the rate of 240 mg. per day for 6 days (2 doses daily), indicate that 78 p.c. of the indole fed was excreted in the urine while approximately 1 p.c. was recovered in the faeces.

The relationship between blood and milk indole levels. Indole in 0·25 gm. quantities was fed to a milking goat at 9.30 a.m., 11.30 a.m., 1.30 p.m. and

3.30 p.m. on each of two successive days. Blood samples were taken at 10 a.m., noon, 2 p.m. and 4 p.m. The results, shown in Fig. 5, reveal that high blood indole contents were obtained, and further, that these resulted in high milk indole levels.

The influence of Lepidium *in the diet on the blood and milk indole levels.* The results given in the graph of Fig. 6 show that when the goat was feeding on a normal diet there was little free indole in either the milk or blood. When *Lepidium* was introduced into the diet the blood indole levels rose sharply during the day and fell towards evening. Milk drawn in the afternoon contained much more free indole than that drawn in the morning. The animal was fed at 9.30 a.m. and 4.30 p.m. and milked at 9 a.m. and 5 p.m.

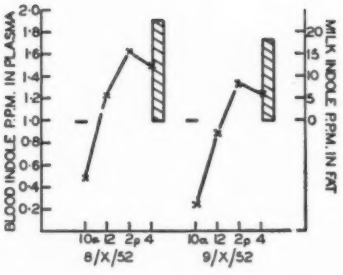

Fig. 5. Showing the blood and milk indole levels attained by giving 1 gm. indole per day by mouth to goat No. 3 on a lucerne diet.

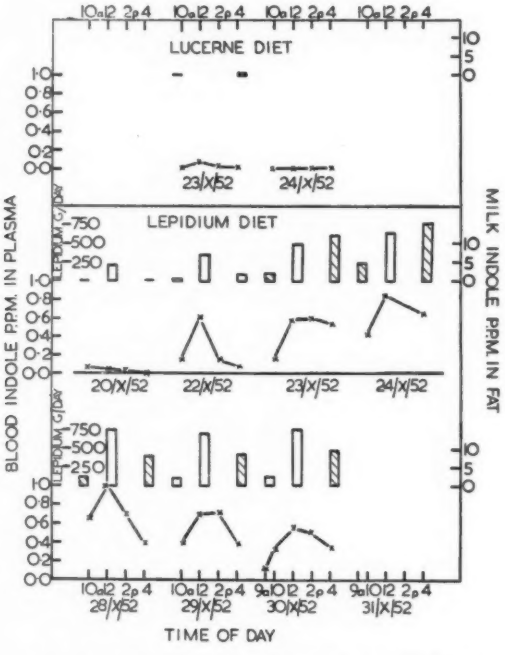

Fig. 6. Showing blood and milk indole levels of goat No. 3 feeding on lucerne and *Lepidium*-containing diets.

Open panel indicates *Lepidium* consumed.
Shaded panel indicates milk indole levels.

The influence of the interval between feeding and milking on the indole content of the milk. In areas where cows feed on *Lepidium* spp. it is observed that the concentration of tainting substances is lower in the morning milk than in

the afternoon milk. This fact was confirmed in the experiments with the goat, as will be seen from Fig. 6.

This same effect was also apparent on week-ends during the goat feeding experiments when the animals were milked in the morning only. It will be seen from Table 7 that the indole content was much lower than when milking twice daily was practised and the day's milk bulked for analysis.

TABLE 6.

The excretion of indole given by mouth (Goat 2).

No indole			Indole at 240 mg./day		
Date 1950	Urinary indican (expressed as gm. indole/day)	Faecal indole (mg./day)	Date 1950	Urinary indican (expressed as gm. indole/day)	Faecal indole (mg./day)
9/11	0.37	5.7	16/11	0.50	5.2
10/11	—	—	17/11	0.53	6.1
11/11	0.43	6.5	18/11	0.62	11.3
12/11	0.46	—	19/11	0.65	10.9
13/11	0.42	3.9	20/11	0.69	8.3
14/11	0.41	4.0	21/11	0.61	7.8
15/11	0.39	5.8			
Average	0.41	5.2	Average	0.60	8.3

TABLE 7.

Indole content of milk drawn once and twice daily expressed as p.p.m. in fat.

Twice		Once	
Date 1949		Date 1949	
29/7	2.15	30/7	0.95
1/8	0.95	31/7	0.50
5/8	9.2	6/8	2.50
8/8	2.7	7/8	1.00
12/8	5.15	13/8	0.90
15/8	5.40	14/8	1.35

The indole and skatole content of milk with Lepidium *taint.* Separate values for the indole and skatole content of milk were obtained in a number of cases. Two samples of herd milk from cows feeding on *Lepidium* under field conditions gave the following figures:

Sample	Indole p.p.m. in fat	Skatole p.p.m. in fat
a	10·4	10·3
b	4·4	5·1

For goats feeding on a diet containing *Lepidium* the figures were:

a	2·8	4·6
b	2·2	4·6
c p.m.	7·2	13·2
d a.m.	3·4	3·9
e p.m.	11·0	15·0
f p.m.	11·3	18·7
g p.m.	5·3	8·1
h p.m.	6·2	6·0
i p.m.	7·4	6·3
j a.m.	2·1	1·3

The presence of both indole and skatole is of interest because Woods (1935) found that washed suspensions of *Esch. coli* gave a quantitative production of indole from tryptophane under aerobic conditions. The method he used for estimating indole was specific for indole.

Samples of *Lepidium*-tainted butter examined showed the presence of both indole and skatole.

DISCUSSION.

Most feed flavours arise from the transfer to the milk through the blood stream of substances such as essential oils, derived directly from the plant material in the alimentary tract. The flavour and aroma of *Lepidium*, however, are in no way suggestive of indole and skatole, and steam distillation of the plant failed to yield any significant concentration of substances giving a reaction for indole.

The most probable parent substance for indole production is tryptophane. The leaves of *Lepidium* were found to contain from 27 to 31 p.c. of protein, against 23 p.c. for the lucerne used in control feeding, and the protein was of normal tryptophane content. It was clear that the tainting of milk by *Lepidium* must be due to some cause less direct than other feed taints.

If the indole and skatole present in the milk of animals feeding on *Lepidium* were derived from tryptophane by the action of coliform organisms, the feeding of antibiotics should have prevented the appearance of indole and skatole in the milk. The treatment, however, was not effective.

Indole production by *Esch. coli* is an aerobic process (Woods, 1935). Rogers (1949) found oxygen pressures of 4 mm. Hg. near the mucosa of the small intestine of sheep, and considered that pressures in the mass of the intestinal contents would be much lower. In the large intestine even more highly anaerobic conditions are to be expected. Under such conditions organisms other than *Esch. coli*, possibly *Clostridium bifermentans* and *Cl. tetani*, or some strains of *Proteus vulgaris*, may be responsible for indole formation. Dr. Hayward suggested that the organisms appearing on the anaerobic blood agar plates when phthalylsulphathiazole was administered may have been *Proteus vulgaris*. A further point which may be of significance is the fact that Woods (1935) found a quantitative formation of indole from tryptophane with washed cell suspensions of *Esch. coli* under aerobic conditions, whereas in milk from animals feeding on *Lepidium* there was at least as much skatole as indole.

Since our work was completed Wooldridge *et al.* (1950) have published the results of a study of indican excretion in relation to "intestinal sterilization" in man. They found that indican excretion was little influenced by treatment with streptomycin and glucoronolactone sufficient to eliminate *Esch. coli*, *Enterococci* and *Clostridia* from the faeces. They concluded that "it does appear necessary to seriously question the accepted theory of 'intestinal putrefaction' caused by the degradation of tryptophane by *Esch. coli*." Our investigations not only support a similar conclusion for the ruminant, but suggest that the whole question of the metabolism of indole and skatole in the ruminant has not been adequately studied.

It was shown that high blood indole levels caused abnormally high levels of milk indole and further that when the animal was changed from a lucerne

diet to a *Lepidium*-containing diet, both blood and milk indole levels rose substantially above normal. Indole is much more soluble in fat than in aqueous media, and the occurrence of high concentration in the blood at the time of fat formation in the mammary gland would result in the building up of only slowly reversible high concentrations in the secreted milk fat. This would apply equally well to skatole, which is even less soluble in aqueous media. The indole content of the milk drawn once daily and twice daily indicated that reabsorption of indole from the milk back into the blood did take place slowly during the period when the animal was not feeding.

There was no evidence to suggest that *Lepidium* caused a greater production of indole and skatole in the alimentary tract, nor that it markedly affected the total daily excretion of indole (urinary indican plus faecal indole). However, a study of the rate of indican excretion throughout the day showed that *Lepidium* did interfere with the excretion of indole as indican, particularly when the animal was feeding. On a normal diet the animal was capable of detoxicating 25 mg. indole per hour, while on *Lepidium* the rate did not exceed 15 mg. per hour, even though free indole was present in the blood. On a lucerne diet 49 p.c. of the daily output was excreted during the period 9 a.m. to 5 p.m. and only 34·5 p.c. for the same period on a *Lepidium*-containing diet.

Further evidence that *Lepidium* interfered with the normal detoxication of indole was given by the observation that, while the high milk indole level obtained by feeding indole did not persist beyond the afternoon milking, milk taken the morning after feeding *Lepidium* contained much indole.

No evidence was obtained as to the way in which *Lepidium* interfered with the detoxication of indole. The plant was not palatable to the goats and produced mild scouring when first introduced into the diet of one animal. Pammel (1911) lists several *Lepidium* species as fish poisons and irritants.

It is not possible to suggest, from the results of this investigation, any treatment for the prevention of tainting by *Lepidium*.

SUMMARY.

The taint in the milk of cows feeding on *Lepidium* spp. was due to the presence of indole and skatole. Analysis of the plant revealed no abnormal amount of any known indole precursor. There was no significant increase in the daily urinary indican excretion nor in the output of faecal indole when *Lepidium* was fed to goats. However, figures for the hourly excretion of urinary indican showed that there was a temporary interference with the detoxication of indole which could be responsible for the high indole content of the milk.

The blood indole content of a goat rose from zero to 1·0 p.p.m. in the plasma when the animal was changed from a lucerne diet to one containing *Lepidium*. It was shown that high concentrations of indole in the blood at the

time of milk secretion caused high concentrations in the milk fat. Transfer of indole back into the blood stream was found to be slow. A high proportion of the indole produced in the alimentary tract was absorbed.

The feeding of antibiotics eliminated *Esch. coli* from the faeces and reduced, but did not prevent, the abnormal concentrations of indole and skatole in the milk. The results cast doubt on whether putrefaction of intestinal contents by *Esch. coli* is the source of the indole normally excreted by the animal as urinary indican. The results do not indicate any practicable means of reducing the incidence of *Lepidium* taint.

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AMMONIA, UREA AND URIC ACID CONTENT OF TOENAILS IN RENAL INSUFFICIENCY AND GOUT

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Determinations have already been made on a number of substances present in the aqueous extract of toenails (non-keratins) obtained from patients, who have died from a wide variety of diseases excluding renal disorders (Bolliger and Gross, 1953). Similar measurements for patients dying with markedly elevated blood urea levels are now reported. For this group, however, the determinations are confined to nitrogenous non-keratins such as ammonia, urea and uric acid. Findings on the nitrogenous non-keratins of toenails, pubic and head hair of a patient with markedly elevated blood urea suffering primarily from chronic gout are also presented. Furthermore, the uric acid content of toe and fingernail parings of normals and of three individuals suffering from gout without renal involvement was also determined.

METHODS.

As described previously (Bolliger and Gross, 1953), the toenails were scraped clean with a scalpel. The analytical methods employed were those already mentioned, the blood urea content being determined a few days before the patient died and sometimes also on prior occasions.

RESULTS.

The analytical results yielded by the toenails of eleven patients with elevated blood urea values are listed in Table 1, together with the corresponding ranges obtained from toenails of some 20 cases published previously which did not exhibit markedly elevated blood urea values or severe renal damage on *post-mortem* examination.

In every instance the sum of urea and ammonia nitrogen was definitely higher than 75 mg. p.c., which is the highest finding in the group of so-called normal reference figures obtained from patients dying from a wide variety of diseases but without severe urea retention. This elevation is due solely to the elevated urea nitrogen content, (62–273 mg. p.c.) because the ammonia content wherever determined lies within normal limits.

The uric acid content of toe and fingernail parings from living normals

[1] Working under a grant from the National Health and Medical Research Council.

varied from 4 to 6 mg. p.c. (Table 2). Nail parings obtained from ambulant patients suffering from gout without elevated blood urea, yielded uric acid values definitely in excess of the upper normal value of 6 mg. p.c. (Table 2). In one instance uric acid content of the toenail was more than twice that found in normals. In a patient (case 11) with numerous large gouty tophi and dying from interstitial nephritis, the uric acid content of toenails, head and pubic hair was about 4 times as high as that of normals (26–28 mg. p.c.). In two patients suffering from longstanding glomerulonephritis and in a case of advanced nephrosclerosis terminating in cardiac failure the uric acid values were 14 mg. p.c. (Cases 1, 2, and 4). Short-lived renal insufficiency with high terminal blood urea nitrogen content yielded normal uric acid level in toenails (Cases 8, 9 and 10).

DISCUSSION.

Urea.

The toenails from eleven patients exhibiting renal insufficiency showed a marked increase in their urea content (Table 1) in comparison with some 20 sets of toenails originating from patients dying from a variety of other diseases (Bolliger and Gross, 1953). In three cases of chronic glomerulonephritis (1, 2 and 3), the urea content of the toenails was about three-quarters of that encountered in the blood. In the other 8 patients examined it is difficult to discern a parallelism of any kind between the blood and toenail urea content. This is partly due to the fact that the specimens of blood for the final urea determination, as listed in Table 1, were taken one or several days before death, which may have been due to uraemia, cerebral haemorrhage, cardiac failure, inter-current infection, etc. Consequently, the terminal rise in blood urea of the types of renal insufficiency examined is of varying magnitude. For example, in a patient dying from complete urinary obstruction (Case 10) the toenail urea level was only about one-third of that of the blood, owing to the lag in the urea accumulation in keratinous tissue compared with the much quicker response of the blood level.

In another patient (Case 4) the urea nitrogen content of the toenails was definitely higher than that of the blood, probably because the blood urea level was reduced by therapeutic measures shortly before death.

The increase of the urea content of the keratinous appendages in renal insufficiency is probably best explained by a spilling over of the elevated urea content of the neighbouring tissues into the keratinous appendages, urea being readily soluble and easily diffusible. Such a process might be expected to require only a few days and the transference of urea into structures such as toenails seems to take place along the entire nailbed. Renal insufficiency with its characteristic retention of urea in blood and tissues thus also has a definite effect on the urea content of the keratinous appendages such as toenails and also hair as has been shown in one of the cases (11).

TABLE 1.

Urea, ammonia and uric acid content of toenails of patients who died with severe renal insufficiency including one with gout.

Case No.	Diagnosis	Age	Sex	Urea + Ammonia N. mg. p.c.	Urea N. mg. p.c.	Ammonia N. mg. p.c.	Uric acid mg. p.c.	Blood—Urea N. mg. p.c.
1.	Chronic glomerulonephritis	51	M.	180	140	40	14	200
2.	Chronic glomerulonephritis	44	F.	191	160	31	14	215
3.	Chronic glomerulonephritis			125	86	39	11	111
4.	Malignant hypertension; nephrosclerosis	44	F.	200	152	48	14	121
5.	Malignant hypertension; nephrosclerosis	65	M.	118			9	60
6.	Nephrosclerosis	64	F.	98	62	36	6	58
7.	Pyelonephritis; tubular necrosis	34	F.	299	273	26	10	298*
8.	Pyelonephritis; tubular necrosis	69	F.	119			6	148
9.	Pyelonephritis; cortical abscess	74	M.	131	92	39	6	134
10.	Urinary retention	84	M.	97			5	182
11.	Gout; interstitial nephritis. Toenails	35	M.	174			26	122
	Head hair			318			28	
	Pubic hair			259			26	
	Deceased "non nephritics"			44–75	15–50	14–50	6–9	
	Living normals						4–6	

* This patient had a blood urea nitrogen exceeding 150 mg. p.c. six weeks and exceeding 250 mg. p.c. two weeks prior to death.

Ammonia.

The ammonia content of the toenails did not exceed "normal" limits in cases with elevated blood urea. Ammonia values seemed to remain at a fixed range in toenails even in conditions of prolonged severe urea retention in the blood and in the keratinous tissues. This confirms rather strikingly that the ammonia found in the keratinous tissues in all probability is manufactured in the keratin-producing structures. Ammonia, a toxic metabolite which is usually converted in the tissues into other less toxic substances can be tolerated in the keratinous structures. It is ultimately "excreted" from the body by the process of shedding of these structures. The analogy with the ammonia formed in the kidney and immediately excreted in the urine is obvious. The occurrence of ammonia in keratin is a further indication of the autonomous nature of keratin-producing structures as was indicated previously by the presence of pentose and by a high amount of uric acid in ureotelic mammals (Bolliger and Gross, 1952b).

In those instances where separate ammonia determinations could not be executed, the combined urea and ammonia nitrogen values are nearly as informative as separate urea nitrogen figures since the ammonia content of toenails is practically a fixed value. In our present series of patients with urea retention, the combined urea and ammonia nitrogen always exceeded 75 mg. p.c., this being the upper limit encountered in patients without marked urea retention.

Uric acid.

The uric acid content of the toenails of patients with renal insufficiency furnishes a different picture. Even with a markedly elevated urea content the non-keratin uric acid may still be within normal limits (Cases 8, 9 and 10). This occurs in acute, short-lived forms of renal insufficiency primarily due to infection and developing within a few days or weeks before death. An increase of the uric acid content could hardly be expected in such a short time because any additional uric acid which may be present in the circulation could in all probability only be deposited at the proximal ends of the toenail root where the keratin formation occurs. In longstanding chronic nephritis, however, the uric acid content of the toenails is considerably increased, reaching values about twice as high as that of normals (Cases 1, 2 and 4).

Figures obtained with Folin's "direct" method of uric acid determination for elderly males with mild or doubtful gout suggested that the uric acid content of head and pubic hair might be increased in this metabolic disturbance (Bolliger, 1947). We were able to obtain not only head and pubic hair but also toenails from a cadaver of a patient who had been suffering with severe gout for at least 10 years. Due to the renal insufficiency prevailing in the last few months of life this patient had a markedly elevated blood urea content as well as a very high urea content in his head and pubic hair and toenails (Case 11).

Uric acid (determined by the specific uricase method) was found to be 26 mg. p.c. in toenails and pubic hair and 28 mg. p.c. in head hair, approximately four times as high as the uric acid content of the toenail of the normal and also about four times as high as the uric acid content of the blood (6 mg. p.c.) shortly before death.

TABLE 2.

Uric acid content of toe and fingernail parings and of head hair of gouty males in quiescent periods and without urea retention and of normal males.

Uric acid determined by the uricase method.

Age	Clinical diagnosis		Uric acid mg. p.c.
64.	Moderately severe gout	Toenails	14
		Fingernails	13
		Head hair	14
61.	Moderate gout	Toenails	11
		Fingernails	11
		Head hair	11
45.	Mild gout	Toenails	9
		Fingernails	8
		Head hair	10
54.	Normal	Toenails	6
		Head hair	5
17–24.	Normal	Toenails. Pooled	5
21.	Normal	Toenails	4

For the estimation of the non-keratin uric acid content, 50 mg. of nail are required and this amount can readily be obtained by means of nail parings from living subjects. Thus, it became possible to evaluate the uric acid content of toe and fingernails and of head hair of 3 patients suffering from gout uncomplicated by renal insufficiency. They all showed definitely increased uric acid values in nails and head hair (Table 2), while their blood uric acid content was within normal limits at the time.

This observation suggests that uric acid determinations on the readily available keratinous appendages and particularly toenails may be of diagnostic value. This aspect will be examined on a larger group of sufferers from gout.

The elevated uric acid values found in gout and to a lesser extent in chronic renal insufficiency could be due either to a spilling over from other tissues into the keratinous structure as such, or in part, at least, to a metabolic disturbance within the keratin-producing structures.

The deposition in the keratinous structures of uric acid which is difficult to excrete when there is renal impairment, seems feasible, particularly since the interesting capacity of keratinous tissues to contain or fix comparatively large amounts of uric acid has repeatedly been demonstrated in other mammals. In nephritic subjects uric acid formed as an end product of the nucleic acid degradation in the process of keratinization (Bolliger and Gross, 1952) may thus be forced into the developing keratin to a greater extent than usual. In advanced gout, moreover, considerably higher uric acid values can be obtained in toenails and hair (Case 11) than in chronic nephritis, though the blood uric acid levels are about the same in the two conditons. At present we are inclined to favour the view that increased deposition in gout is not due to a metabolic disturbance within the keratin-producing structures but is simply an excretory mechanism.

It should be pointed out, however, that the uric acid levels in human nails and hair in health and even in disease, including severe nephritis and gout, are still low compared with the uric acid content encountered in the keratinous appendages of the integument of many other mammals (Bolliger and Gross, 1952, 1952a), although these animals are not known to suffer from gout in any significant numbers. One may be permitted, therefore, to speculate on a possible connection between gout, which is a condition peculiar to human beings, and the low urate content of the sparse human hair and of other keratinous appendages. This means that in the human subject comparatively little uric acid is excreted by the integumentary route, so that a consequent tendency for urates to deposit in the subcutaneous tissue is to be expected. It may be significant that the pig with a sparse keratinous integument of low uric acid content has been reported to suffer occasionally from a type of guanine gout.

SUMMARY.

The toenails of eleven patients with varying types and degrees of renal impairment, but all showing markedly increased blood urea levels at the time of death, were examined for water extractable urea, ammonia and uric acid.

The urea content of these toenails was always elevated.

The ammonia content did not exceed normal values.

The uric acid content of the toenails was markedly elevated in patients with chronic nephritis but was normal in renal impairment of short duration.

In a patient with long standing severe gout dying from interstitial nephritis the uric acid content of the toenails was about four times as high as that from normals. The uric acid content of nail parings from three living patients suffering from gout without definite renal involvement was increased.

The relation of these findings to the problem of gout is discussed.

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STUDIES ON THE SOLUBLE COMPLEMENT FIXING ANTIGEN OF INFLUENZA VIRUS

II. SEROLOGICAL BEHAVIOUR OF THE ANTIGEN

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In the first paper of this series (Ada, Donnelley and Pye, 1952) methods were described whereby the soluble complement fixing antigen of influenza virus could be isolated from extracts of lungs from 18-day-old embryo chickens which had been inoculated in the amniotic cavity with virus 48 hours previously. These methods involved either fractionation with organic solvents or differential centrifugation in an ultracentrifuge. The activity was found to be associated with particles having a molecular weight of approximately 1,500,000. The electrophoretic behaviour of the preparation suggested a population of particles of similar size and shape. It was realized, however, that such preparations would consist of components from both infected and non-infected cells as it is known that material exhibiting similar physical properties can be isolated by similar means from extracts of normal embryo chick lungs. The possibility of further purification depended then on finding a method which would differentiate between the antigen and other particles. Utilization of the serological specificity of the antigen appeared to be the most suitable method of achieving this.

This report deals with the serological behaviour of the C.F. antigen. For purposes of comparison, preparations have also been made from extracts of normal chicken lungs, infected mouse lungs and normal mouse lungs. Sera used have been obtained from mice convalescing from influenza or from rabbits following inoculation with infected or normal chicken lung extracts. The antigen antibody reaction has been followed by complement fixation tests, micro analytical precipitin tests and/or the "Oudin" ring test in which rapid diffusion of the specific precipitate formed is prevented. As a result, it has been possible to form complexes between the C.F. antigen and its specific antibody and so separate the antigen from the other inactive particles present in the centrifugally purified preparations.

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Materials and Methods.

Virus strains. The classical influenza strains, MEL and SWINE (Type A) were cultivated in the allantoic cavity of 11-day-old hen eggs and the allantoic fluid harvested after 42 hours.

Complement fixation technique. The basic technique was that described previously (Donnelley, 1951). In the block tests, 8 rows of 12 tubes were distributed in racks and 0·04 ml. of complement, previously diluted to 3 HD_{50} added. To the tubes in the first row was added 0·04 ml. of a 1: 5 dilution of antiserum. The next row of tubes contained the same volume of a 1: 10 dilution, the next row a 1: 20 dilution, and so on. To the first tube of each row was added 0·04 ml. of a 1: 10 dilution of antigen. To the next tube of each row was added 0·04 ml. of a 1: 20 dilution of antigen, and so on. The test was then carried out as described previously (Donnelley, 1951).

Definition of unit. A solution is said to have a titre of x units when a dilution of $1/x$ in 0·04 ml. volume used in the C.F. technique described (total volume in the test being 0·16 ml.) gives 50 p.c. haemolysis.

Precipitin block test. Reagents were diluted in 0·5 p.c. (w/v) sodium chloride containing 1,000 units of sodium penicillin per ml. Distribution of antigen and serum was arranged as described in the C.F. block test except that 4 dilutions of serum and 6 of antigen were used. Standard volume of each reagent was 0·5 ml., initial dilution usually being 1: 2. The test was set up under sterile conditions.

Agar. The agar used was a preparation purchased locally (Davis Agar).

Protein estimation. Protein concentration was estimated by the micro method of Folin-Ciocalteu as described by Kabat and Mayer (1948): Results were periodically checked by micro Kjeldahl and a factor of 6·25 assumed.

Ultracentrifugation. High speed centrifugation was carried out either in an air-driven ultracentrifuge which has been described previously (Ada *et al.*, 1952) or in a Spinco Model L ultracentrifuge. In the latter case, rotors No. 21 and 30 were used. These have the following specifications:

No. 21. Angle of tube to axis of rotation 18°.
Distance of mid point in tube to axis of rotation 9·0 cm.
Capacity of tube 94 ml. No. of tubes, 10.

No. 30. Angle of tube to axis of rotation 26°.
Distance of mid point in tube to axis of rotation 7·8 cm.
Capacity of tube 38·5 ml. No. of tubes, 12.

Electrophoresis. Electrophoresis was carried out at 2° C. in cells of 2 ml. capacity in a Tiselius apparatus with Philpot-Svenssen optical system (Holden, Ada and Pye, 1952). Buffers used were:

Glycine $\mu = 0{\cdot}05$, pH 3·0, 0·25 M glycine, 0·05 M HCl.
Phosphate $\mu = 0{\cdot}2$, pH 7·0, 0·091 M KH_2PO_4, 0·055 M KOH.
Veronal $\mu = 0{\cdot}1$, pH 8·6, 0·12 M Veronal, 0·01 M Na OH.
Glycine $\mu = 0{\cdot}2$, pH 10·0, 0·30 M glycine, 0·20 M KOH.
Glycine $\mu = 0{\cdot}05$, pH 11·6, 0·039 M glycine, 0·05 M KOH.
Glycine $\mu = 0{\cdot}2$, pH 11·6, 0·039 M glycine, 0·05 M KOH 0·15 M KCl.

EXPERIMENTAL.

Preparation of antigens.

Infected chicken lung extract. This was prepared as described previously (Ada *et al.*, 1952), with some modifications which were introduced to maintain sterility. Briefly, the lungs were ground with alundum and taken up in 10 p.c. sterile sucrose solution at the rate of 1 ml. per two lungs. The virus present was removed by adsorption onto red cells. The extract was then clarified by centrifugation at 20,000 *g* for 20 minutes. The supernatant was passed through a Hormann D 3 clarifying pad (finer pads retained portion of the activity). The filtrate was layered over a 30 p.c. (w/v) sucrose solution (in the approximate proportion of 1 vol. of sucrose solution under $2\frac{1}{2}$ vols. of filtrate) in a centrifuge tube and centrifuged at 75,000 *g* for 2 hours. The antigen sedimented into the lower sucrose layer and after removing the upper pigmented solution, could be recovered by resuspending the sediment in the sucrose layer. After filtration through a Hormann D 3 pad, the filtrate was labelled ICLE and stored at —20° C. until required. Such preparations contained 0·2–0·3 p.c. protein, the titre being about 500 units/0·1 p.c. solution.

Normal chicken lung extract. This was prepared in exactly the same way as the ICLE. After final filtration, the preparation was labelled NCLE and stored at —20° C. While ICLE preparations were only slightly pigmented, NCLE preparations were markedly coloured. The yield in dry weight was considerably greater in the case of NCLE compared with ICLE, the concentration being in the range 0·5–0·8 p.c. protein.

Infected mouse lung extract. Mice were inoculated intranasally with MEL virus and the lungs harvested on the second day after infection (Donnelley 1951). The method of fractionation employed was similar to that given above except that the extract was not filtered at any stage. After final resuspension, the extract was labelled IMLE, the titre being 30–60 units/1 p.c. (w/v) solution.

Normal mouse lung extract. Extracts from normal mouse lungs were purified as in the case of IMLE, and labelled NMLE.

Electrophoretic behaviour of the antigens.

The results of electrophoresis of ICLE have already been published (Ada *et al.*, 1952) when it was shown that, although the preparation exhibited a single peak in some buffers, it was heterogeneous. Electrophoretic patterns of NCLE in phosphate pH 7·0, $\mu = 0{\cdot}2$, veronal pH 8·6, $\mu = 0{\cdot}1$, and glycine pH 10·0, $\mu = 0{\cdot}2$, were similar to those of ICLE, indicating that it also is heterogeneous, the mean mobility being of the same order. However, the range of mobilities seems to be appreciably greater in the case of NCLE.

NMLE and IMLE both displayed very diffuse single peaks in phosphate buffer pH 7·0, $\mu = 0{\cdot}2$.

Preparation of sera.

Mouse sera. Mouse convalescent serum was obtained as previously described (Donnelley, 1951). Briefly, the mice were inoculated intranasally with MEL virus (10^{-6} dilution, 0·05 ml.) and were bled on the 12th day after infection. The serum was removed from the contracted clot, heated at 56° C. for 30 minutes, sterilized by filtration through a Hormann D 9 pad and stored at —20° C. until required.

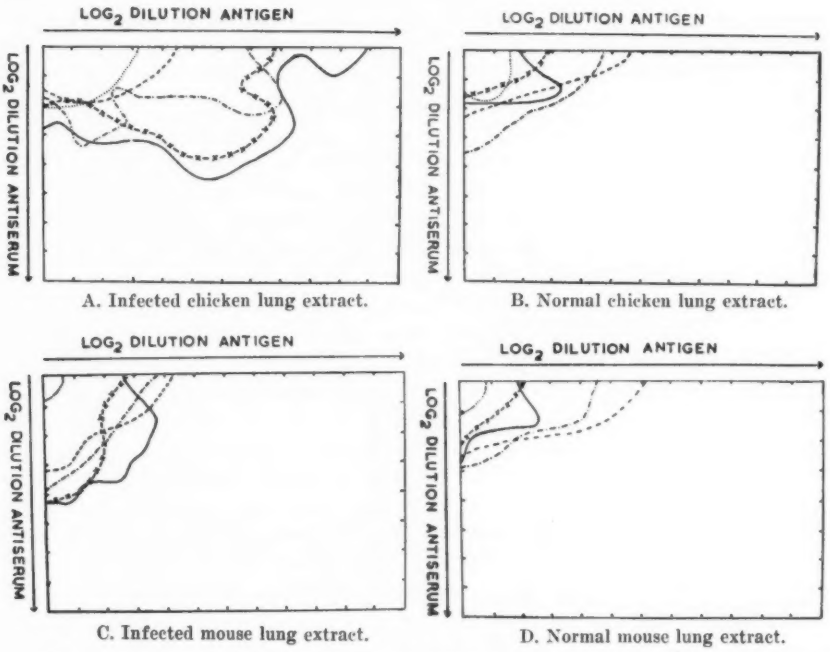
LOG2 DILUTION ANTIGEN
LOG2 DILUTION ANTISERUM
A. Infected chicken lung extract.
B. Normal chicken lung extract.
C. Infected mouse lung extract.
D. Normal mouse lung extract.

Fig. 1. The extent of antigen-antibody reaction as shown by complement fixation between anti-infected chicken lung extract—Rabbit serum and A. Infected chicken lung extract; B. Normal chicken lung extract; C. Infected mouse lung extract; D. Normal mouse lung extract.

Reaction with serum taken

0 days after inoculation .....
5 days after inoculation - - - - - - - - - -
10 days after inoculation -.-.-.-.-.-.-.-
15 days after inoculation -x-x-x-x-x-
20 days after inoculation ————

Rabbit sera. Antibodies were prepared in rabbits to ICLE and NCLE. As other workers (eg. Freund *et al.*, 1948) have obtained high antibody levels which persisted for a long period following the use of antigen combined with an adjuvant, a similar technique was adopted. Two volumes of antigen were mixed

with 2 volumes of paraffin oil and 1 volume of lanoline was used as the emulsifier. Rabbits were given simultaneously 2 subcutaneous inoculations of 0·7–1·0 ml. each of the mixture and were bled from the ear vein at the required intervals. The serum was removed from the clotted blood, inactivated at 56° C. for 30 minutes, filtered through a Hormann D 9 pad and stored at —20° C.

Complement fixation block tests. Complement fixation offers a rapid method for determining the amount of antigen or antibody present in a system. By carrying out a "block" test in which varying concentrations of antigen and antibody are mixed, an overall picture of the extent of antibody-antigen reaction in a system is obtained. The technique has been used in this investigation largely for the purpose of comparing the production of antibody in the serum at various stages after the inoculation of antigen. Details of the technique are given in Materials and Methods.

For the sake of brevity, the results are not presented in the usual fashion in which the degree of lysis in each tube is represented as a symbol. After recording each test in the usual way, a line has been drawn through those values representing 50 p.c. lysis. The shape of each curve and the area under it then indicate the amount of antigen-antibody interaction in a test. In Fig. 1 several curves are superimposed in order to facilitate comparison.

Four rabbits were inoculated subcutaneously with ICLE. Samples of blood were taken on the 5th, 10th, 15th and 20th days after inoculation and C.F. block tests carried out on the serum using all four antigens. The results from one rabbit presented in Fig. 1 are typical. The curves indicate the extent of lysis—using antigens which have been adjusted to the same N concentration. Clearly, there is a marked reaction in the case of ICLE, less marked with IMLE, less still with NCLE and very little reaction with NMLE.

Comparison of the curves obtained with the four rabbits shows the following. In the case of NCLE and NMLE, maximum antibody titre occurs on the 10th day and then declines fairly rapidly. Maximum titres with ICLE and IMLE are not reached till the 15th day and remain high until (at least) the 20th day. Seven weeks after the 1st inoculation, reaction with ICLE had decreased while that with NCLE had virtually disappeared.

At this stage, a second similar inoculation was given. Twelve days later the rabbits were bled and the sera tested. Compared with sera obtained 20 days after the first inoculation the reaction with ICLE was slightly higher while that with NCLE showed a correspondingly greater increase. Fifty days after the second inoculation, however, the reactions with ICLE and NCLE were similar to those with 20 day sera following the first inoculation.

Four rabbits were inoculated with NCLE in a similar fashion. Samples were taken on the 5th, 10 and 20th days and tested. Responses were so slight that no satisfactory comparison could be made between the reactions with the different

antigens. Five weeks later, a second inoculation was given. Serum obtained 13 days later showed little increased reaction with any of the antigens.

Mouse convalescent sera were also tested by this technique against ICLE and NCLE. NCLE showed no reaction at all while ICLE gave a marked reaction, the pattern of which was very similar to, though slightly weaker than, that given by ICLE with anti-ICLE serum (20th day bleeding after 1st inoculation) (see Fig. 2).

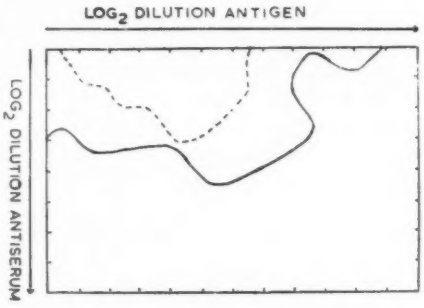

Fig. 2. The extent of antigen-antibody reaction as shown by complement fixation between infected chicken lung extract and
Anti-infected chicken lung extract-rabbit serum———
Mouse convalescent serum - - - -

Antigen-antibody reactions in agar.

The modification of the original ring test introduced by Oudin (1946) and subsequently used by several other workers has also been used in this investigation to compare with reactions of sera with different antigens. In this method, serum containing antibody is mixed with gelatine or agar to form a gel. The antigen solution is then brought into contact with the antibody gel so that diffusion of the components of one into the other is possible. Generally, a high concentration of antigen is chosen with a relatively lower concentration of antibody, so that the resulting formation of specific precipitate takes place in the gel which then prevents rapid diffusion of the precipiptate.

The technique used was that described by Munoz and Becker (1950). Briefly, the reaction is carried out in glass tubes (approx. 5 cm. long, bore 3 mm.) which are sealed at one end. Serum at 50° C. is mixed with an equal quantity of 0·3 p.c. agar in distilled water and the tube half-filled. After the agar has set, the antigen solution is layered over the serum and the tube plugged with plasticine to prevent evaporation.

Antigens were used in the state prepared by ultracentrifugation except for the addition of sodium penicillin to give a final titre of 1,000 units/ml. All reactions were carried out at 2° C. as it was found that the antigens were unstable at higher temperatures. The tubes were examined at two day intervals over periods of 2–3 weeks.

The results obtained with sera from four rabbits taken at intervals after inoculation with ICLE are summarized in Table 1. Strong reactions were given by ICLE and IMLE wih sera taken on the 10th, 15th and 20th days after the first inoculation. A faint precipitate which occurred with the sera from the 20th day bleeding from one rabbit was the only indication of any reaction with NCLE. NMLE gave no reaction with any serum.

TABLE 1.

Antigen-antibody reactions in agar between purified antigens and rabbit sera taken at varying intervals after inoculation (with adjuvants) of ICLE.*

No. of inoculations	Days after inoculation	No. of rings present after 2 weeks at 2° C. with the antigens ICLE*	NCLE*	IMLE*	NMLE*
1	5	0	0	0	0
	10	4–5	0	3	0
	15	5	0	3–5	0
	20	4–5	0–1†	3–4	0
2	12	5–9	3–5	4	0
	50	4	0	2–4	0

* Figures give the range of reaction in sera taken from four rabbits.
ICLE—infected chick lung extract. IMLE—infected mouse lung extract.
NCLE—normal chick lung extract. NMLE—normal mouse lung extract.

† Very faint precipitation in one tube, appearing between 1st–2nd week.

Seven weeks after the first, a second inoculation of ICLE was given and blood samples taken 12 and 50 days later. Sera from the 12th day bleeding showed an increased reaction with ICLE and marked reactions with NCLE and IMLE. By the 50th day, the reaction with NCLE had disappeared while that with ICLE diminished to the level shown with sera from the bleeding on the 20th day, after the first inoculation. It is not possible to say definitely whether the increase in the number of rings given by ICLE with 15-day sera after the second inoculation is due to increased antibody formation to the C.F. antigen itself, or to components in the ICLE originating from uninfected cells. The reaction shown by NCLE may be due to the latter or to cross reactions with C.F. antigens which show up when there is a sufficiently large concentration of the antibody.

Similar reactions were also carried out with sera from rabbits inoculated with NCLE (see previous section) The results may be summarized as follows. Serum taken 12 days after the first inoculation from 2 out of 4 rabbits gave faint precipitates with ICLE and NCLE but not with IMLE or NMLE. The other two sera showed no reaction. Sera from all four rabbits taken 13 days after a second inoculation reacted with ICLE and NCLE but not with IMLE or NMLE. In each case the rings were ill-defined, rather faint and very slow in forming. There appeared little difference in intensity between the rings formed with ICLE or NCLE.

Mouse convalescent serum was also tested. Both ICLE and IMLE gave marked precipitates which as a general rule were very diffuse, thus preventing an estimation of the number of components. NCLE showed no reaction.

Comparison of results.

Bearing in mind that complement fixation is the more sensitive of the two techniques used, the results obtained agree quite well and clearly indicate that

if ICLE is used as an antigen, it is possible to obtain sera from rabbits which react specifically to components from infected cells. Whether the C.F. antigen is the sole component concerned is not known. The definition of C.F. antigen adopted is that material, excluding components capable of adsorption onto red cells, which will specifically react with convalescent sera. For reasons such as ready availability and high specificity (Donnelley, 1951), mouse serum has been used. The complement fixation patterns of mouse convalescent sera and rabbit sera obtained 20 days after a single inoculation of ICLE, when tested against ICLE, show a striking similarity. In addition, both sera show strong reactions with ICLE when tested by the Oudin technique although sharp rings are given by rabbit serum compared with a diffuse precipitate by the mouse serum.

A possibility remained that some of the reaction between ICLE and the rabbit sera was due to the presence of particulate virus in the ICLE, resulting in the formation of antibody to the virus in the anti-ICLE sera. Indeed, when tested for the presence of antibodies inhibiting haemagglutination of MEL virus, the anti-ICLE sera gave a 2–4 fold higher titre than normal rabbit sera. In order to assess the extent to which this reaction contributed to that between ICLE and the rabbit anti sera, an ICLE solution was prepared from lungs of chickens previously inoculated with Swine influenza virus (Sw). This strain was used because serologically it is distinct from MEL in the anti-haemagglutinin test but yields an identical soluble C.F. antigen. When tested by complement fixation and by the Oudin technique against both rabbit anti-ICLE serum (MEL) and mouse convalescent serum (MEL), the Sw-ICLE and MEL-ICLE gave identical results, thus showing that the reaction between particulate virus and antibody formed to it in the anti-ICLE rabbit sera did not materially contribute to the soluble antigen-antibody reaction.

The evidence outlined above was considered sufficient to warrant the preparation of C.F. antigen-antibody complexes as a further step in the purification of the C.F. antigen. As a preliminary, micro analytical precipitin block tests were carried out to determine antigen-antibody proportions for optimal precipitation.

Precipitin block tests.

These were carried out as described in Materials and Methods. After standing for the determined period of time, the tubes were spun in an angle centrifuge at 0° C. for 45 minutes (approx. 2,000 *g*) and the supernatants removed. The precipitates were washed twice with saline and then estimated for protein content.

In early experiments the test was kept at 37° C. for 2 hours, followed by 48 hours at 2° C. It was found that maximum precipitation did not occur under these conditions, however, and that 10–14 days at 2° C. were necessary to obtain

full precipitation. In the experiments to be reported, the mixtures were not incubated at 37° C. but merely kept at 2° C. for a minimum of 10 days.

In Table 2 are given the results of typical experiments using ICLE and NCLE as antigens, and rabbit and mouse sera. Invariably, control tubes containing dilutions of antigen were found to have precipitates. In the case of ICLE maximum values were of the order 1–2 μg. of nitrogen. NCLE proved to be more unstable, however, and in some experiments 4–5 μg. of nitrogen were deposited in the tubes containing most antigen. These were taken as blank values and subtracted from the main values to give those presented in Table 2.

TABLE 2.

Precipitin block tests.

Reaction between anti-infected chicken lung extract—rabbit serum and

A. Infected chicken lung extract

Log_2 Antiserum dilution \ Log_2 Antigen dilution →						
	19	10	3	<1	<1	<1
	5	9	4	<1	<1	<1
	3	3	1	<1	<1	<1
	2	1	1	<1	<1	<1

B. Normal chicken lung extract

Log_2 Antiserum dilution \ Log_2 Antigen dilution →						
	1	1	3	<1	<1	<1
	1	<1	3	1	<1	<1
	<1	2	2	1	<1	<1
	<1	2	2	1	<1	<1

Reaction between sera from mice convalescing from influenza and

A. Infected chicken lung extract

Log_2 Antiserum dilution \ Log_2 Antigen dilution →						
	21	18	5	2	<1	<1
	5	6	3	2	<1	<1
	3	3	3	2	<1	<1
	<1	1	<1	<1	<1	<1

B. Normal chicken lung extract

Log_2 Antiserum dilution \ Log_2 Antigen dilution →						
	<1	2	1	<1	<1	<1
	<1	2	1	<1	<1	<1
	<1	3	1	<1	<1	<1
	<1	4	1	<1	<1	<1

Figures refer to micrograms of tyrosine and trytophane as estimated by the method of Folin and Ciocalteu.

The results clearly confirm the specificity of the sera used and indicate that the C.F. antigen can be isolated equally well using either sera.

Formation of antigen-antibody complexes.

Six antigen-antibody complexes have been formed, four with mouse convalescent sera and two with anti-ICLE rabbit serum (either from 20th day bleeding after first inoculation or 50 day bleeding after second inoculation). Micro analytical precipitin block tests were carried out before each reaction to determine the dilution of antigen and antibody which would give optimum precipitation. The following is a typical experiment.

200 ml. of antigen solution (diluted ¼ from original preparation) were added to 200 ml. of serum (diluted ½ from original serum). Sodium penicillin was added to give a final titre of 1,000 units/ml. The mixture was let stand 10 days at 2° C. The precipitate was then removed by centrifugation at 10,000 *g* for 30 minutes at 2° C. (Spinco, rotor No. 21), thoroughly suspended in saline and resedimented under the same conditions (rotor No. 30). Washing was repeated three times when the final supernatant failed to give any turbidity with either trichloracetic or sulphosalicylic acids. The washings were added to the original supernatant which was then tested for biological activity by complement fixation, giving the following results. Titre with antigen, 3; with serum, 3·3; with saline, 2. The sediment, after drying from the frozen state *in vacuo*, weighed 57 mg.

Other complexes were prepared in a similar fashion and stored either at —10° C. or in the dried state.

Attempted dissociation of antigen-antibody complexes.

Antigen-antibody complexes have been dissociated in the past by treatment with buffers of high or low pH values or by solution in sodium chloride solution of high concentration. These methods were applied to the present problem. Preliminary experiments showed that ICLE was stable (at low temperatures and for short periods) in buffers ranging from pH values 3–3·5 and 6–11·6, and in 15 p.c. (w/v) sodium chloride solutions (pH 7·0). The antigen was unstable at the extreme pH values in the presence of high salt concentrations.

The complexes proved to be partly soluble in glycine buffers of pH 3 and pH 10, and in 15 p.c. sodium chloride solution. Complete solution occurred in a glycine buffer of pH 11·6, however, and such a solution was examined further to determine whether any dissociation had taken place.

Due to the different isoelectric point of the antigen and γ globulin (approximately 5·0 and 6·8 respectively), it was thought that an electrophoretic examination might reveal the presence of two boundaries if dissociation had occurred. When examined in glycine buffer (pH 11·6, $\mu = 0{\cdot}05$) a single boundary occurred in the ascending limb while partial separations into two peaks occurred in the descending limb. Examination in the same buffer to which potassium chloride had been added to increase the ionic strength to 0·2 (a procedure which should minimize protein interaction), a single boundary in both limbs was evident. Electrophoresis was also carried out at pH 3 (glycine buffer, $\mu = 0{\cdot}05$). While the complex is not completely soluble in this medium, a sufficient concentration was obtained for the purpose. Only one boundary in both limbs was apparent.

The solution was also examined in the ultracentrifuge. It was predetermined that, under conditions which sufficed to sediment 90 p.c. of the antigen (75,000 *g* for 2 hours over a 30 p.c. sucrose layer), the fraction of rabbit globulin possessing antibody activity was only partially sedimented and none penetrated the 30 p.c. sucrose layer. If complete or partial dissociation had taken place therefore, it was to be expected that a significant proportion of antibody

in excess of antigen present should remain in the supernatant above the sucrose layer. A solution of the complex at pH 11·6 (4 ml.) was layered over 30 p.c. sucrose (2 ml). and centrifuged as mentioned above. Layers were removed from the tube as previously described (Ada *et al.*, 1952) and after neutralization to pH 7, were estimated for activity by complement fixation. The results showed that dissociation occurred to a slight extent but it was not sufficient to indicate that the C.F. antigen could be satisfactorily separated from the complex in this fashion.

DISCUSSION.

The presence of species specific antibodies in convalescent serum following infection by influenza virus has been tacitly assumed to be due to the stimulus of the soluble C.F. antigen. The present investigation clearly shows that injection into the rabbit of ICLE (containing virus to a haemagglutinating titre of less than 2) will result in the formation of antibodies which react in a very similar fashion, as judged by complement fixation and micro analytical precipitin block tests, to the antibodies present in mouse convalescent serum. This finding materially strengthens the above assumption.

The C.F. antigen *per se* appears to be more antigenic to the rabbit than are components in the NCLE, as injection of NCLE into the rabbit results in a much poorer antibody response compared with that following injection of only one-third the amount of ICLE. The pattern added by virus infection is evidently more intrinsically antigenic than anything on the normal surface of the microsomal unit.

It was originally considered that the C.F. antigen could be prepared by two methods. Either precipitation from mouse convalescent serum or anti-ICLE serum of the C.F. antigen-antibody complex using ICLE as antigen, or precipitation from ICLE of components reacting with anti-NCLE serum, leaving the C.F. antigen in the supernatant. The poor antigenic response to NCLE led us to abandon the latter proposal and concentrate on the former.

It has been generally accepted that, when antigen-antibody reactions are carried out under conditions minimizing the diffusion of the specific precipitate, the presence of more than one ring or line of precipitation indicates the heterogeneity of the antigen system. As more than one ring is formed between ICLE and anti-ICLE rabbit serum (the serum showing no reaction with NCLE) this clearly points to the complex nature of the antigen and suggests that the term should be changed to C.F. antigens. It is not possible to estimate the actual number of components present as the number of rings can only be regarded as indicating the minimum number. Also, no reason can be given why sharp rings occur with rabbit serum compared with a diffuse precipitate in the case of mouse convalescent serum. It may be an indication that the mouse lacks the power which the rabbit possesses to respond differentially to closely related

antigens. In view of the fact that both sera gave similar results with ICLE (complement fixation and precipitin block tests) the ring formations indicate only a minor difference in the antibodies. The suggestion that this would indicate similar differences between C.F. antigens produced in the chick lung and mouse lung is not substantiated by the finding that IMLE reacting with anti-ICLE serum gives rings equally as sharp as with ICLE.

In view of the heterogeneity of the C.F. antigen, the question arises whether the formation of complexes using only a single dilution of antigen and antibody will result in the precipitation of a sufficient proportion of the antigen to justify its subsequent use as a representative sample of all antigen present. If the different components were to show optimal precipitation at widely different proportions, precipitation at one dilution would not give a representative sample of the antigen. Perusal of the figures in Table 2 gives no indication of more than one optimal precipitation zone, however, and this is confirmed by the finding that after removal of the complex by centrifugation, examination of the supernatant shows little antigen or antibody to be present.

Comparatively little work has been done on the dissociation of antigen antibody systems. In most cases, the method has been employed to purify the antibody, and often antigens with some special organic grouping attached have facilitated the dissociation. In a recent series of experiments, Kleinschmidt and Boyer (1952) found that 100 p.c. dissociation of egg albumin-anti egg albumin precipitates occurred only at pH 12·3, at which value the γ globulin was markedly changed. Similarly, exposure of γ globulin at pH 3·4 or less produced changes. In the present case the antigen would be unstable also at the higher pH. It is also possible that the long period of formation makes it more difficult to dissociate the complex. In this connection, Campbell *et al.* (1951) found that antibody which had been combined to an antigen coupled to cellulose could be almost quantitatively dissociated if elution was carried out within 2 hours after formation. When stored in the refrigerator for more than two days, little or no antibody could be eluted.

Despite the failure to split the complex, a significant advance in the purification has been achieved. Due to the absence of nucleic acid in serum proteins, any such components found by analysis in the complex must be part of the C.F. antigen. The results of such analysis will be reported in a later publication.

SUMMARY.

Following inoculation of mice and of 16-day-old chick embryos with MEL or SWINE strains (A) of influenza virus, extracts of the infected lungs have been purified by differential centrifugation. The final preparations, infected mouse lung extract (IMLE) and infected chicken lung extract (ICLE), contain significant proportions of the soluble complement fixing (C.F.) antigen of influenza virus and have been used to study the antigen's serological behaviour. As controls, extracts of normal chick embryo lungs (NCLE) and normal mouse lungs (NMLE) have been similarly purified.

Sera used have been obtained from mice convalescing from influenza (MEL) or from rabbits following inoculation with ICLE or NCLE.

The extent of antibody-antigen interaction has been followed by the three techniques: complement fixation, specific precipitate formations in gels (Oudin ring test) and micro analytical precipitin tests.

Following a single inoculation of ICLE with adjuvants into rabbits, serum is obtained which, when tested against ICLE, reacts in a similar fashion to mouse convalescent serum. Inoculation of NCLE into rabbits results in a very poor antigenic response.

C.F. antigen-antibody complexes have been formed between ICLE and mouse convalescent sera or anti-ICLE rabbit serum. In this way the C.F. antigen has been obtained free from less antigenic components in the ICLE preparation.

It has not been found possible to dissociate the antigen-antibody complex under conditions which would liberate the native antigen Despite this, an analysis of the complex should reveal whether the C.F. antigen contains nucleic acid.

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THE EFFECT OF NORADRENALINE-INDUCED SYSTEMIC VASOCONSTRICTION ON THE FORMATION OF PULMONARY OEDEMA

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In a recent paper Courtice and Korner (1952) found that prolonged anoxia rendered rabbits more susceptible than animals breathing air, to the production of pulmonary oedema by means of massive intravenous infusions of Ringer-Locke solution. They concluded that haemodynamic changes, rather than changes in pulmonary capillary permeability, adequately explained their experimental results. It was found that prolonged anoxia led to a fall in cardiac output and in mean arterial pressure and to a rise in systemic venous pressure. There was also considerable systemic vasoconstriction following five hours' anoxia. It was not possible to decide whether the relative increase in pulmonary blood volume which rendered rabbits more susceptible to overtransfusion was the result of interferfence with the dynamic blood flow due to relative left ventricular failure, or was due to vasomotor redistribution of blood owing to systemic vasoconstriction. It seemed likely that in the presence of such a degree of cardiac failure as was present in the experiments both these factors probably played a part.

The present study was undertaken to investigate the rôle played by systemic vasoconstriction in the absence of cardiac failure in the production of pulmonary oedema. The standard transfusion method for producing pulmonary oedema previously described (Courtice and Korner, 1952) was used in this investigation. In the experiments in which noradrenaline was administered to the animals, the amount of noradrenaline which was dissolved in the Ringer-Locke solution was so adjusted that each animal received a constant amount per kilogram per minute during the five hour infusion period. Noradrenaline was chosen as the vasoconstrictor agent in this study because in physiological doses it is known to have little effect on the cardiac output in man (Goldenberg, Pines, Baldwin, Greene and Roh, 1948); with the exception of its dilator effect on the coronary circulation (Burn and Hutcheon, 1949) it has predomin-

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antly constrictor properties on the various systemic vascular beds such as skin, muscle, brain and kidney (v. Euler, 1950; Swan, 1949; Moyer and Handley, 1952).

Methods.

Rabbits, varying in weight from 1·4–2·6 kg. were used in all experiments which were carried out on unanaesthetized animals.

Transfusion. Twenty-four rabbits given noradrenaline, as well as 16 control animals, were transfused through polyethylene tubing inserted into their jugular vein. The operative technique and general experimental procedure have been fully described elsewhere (Courtice and Korner, 1952). After operation 1–2 hours were allowed to elapse till the rabbits emerged from their anaesthetic. At the end of that time they were suspended in a rabbit sling and the transfusion of sterile Ringer-Locke solution was commenced. In the experiments in which l-noradrenaline ("Levophed": Stearns) was administered, rabbits were infused with varying amounts of fluid, but always given a constant dose of noradrenaline. In most experiments this dose was 0·3 μg./kg./minute, but in a few experiments larger doses were used. In addition to the 16 operated controls, a further series of 18 controls performed on unoperated animals was carried out. In these, Ringer-Locke solution was administered through a 23-gauge hypodermic needle inserted into their ear vein. As the responses of the operated and unoperated controls were practically identical, their results have usually been pooled. All transfusion experiments were of five hours' duration.

Arterial and venous pressures and heart rates. In all animals given noradrenaline and in the operated controls, arterial pressures were recorded through the carotid polyethylene tube by means of a Hürthle type of manometer. Venous pressures were measured through the jugular polyethylene catheter by a modification of the method described by Burch and Winsor (1943). In the unoperated controls arterial pressures were not measured and the venous pressures were measured in the ear vein by the method used above. Heart rates were determined by listening with a stethoscope and timing it with a stopwatch—the mean of three successive 10-second periods was taken as the heart rate at the time.

Cardiac output determination and air and blood gas analyses. In six animals subsequently given noradrenaline an initial resting cardiac output was determined on unanaesthetized rabbits 2 hours after operation. After this cardiac output determination half an hour was allowed to elapse before commencing transfusion, to allow arterial and venous pressures to become stabilized. The final samples were obtained at the end of five hours' transfusion. In order to obtain reliable blood pressure and cardiac output figures for the final samples, the half-hour waiting period after the first blood samples is essential, as rabbits tolerate the infusion of noradrenaline very badly unless the blood pressure has become stabilized following the first samples. Mean systemic arterial and jugular venous pressures were recorded simultaneously with the cardiac output determinations. Exactly the same procedure was followed in six control animals.

RESULTS.

Effect of noradrenaline on distribution of infused fluid.

The effect of noradrenaline-containing Ringer-Locke infusions on secretion of urine. In Fig. 1(A) are plotted the results of urine passed for varying amounts of Ringer-Locke solution infused for five hours. It may be seen that animals given noradrenaline at the rate of 0·3 μg./kg./minute secreted a

greater amount of urine during the five-hour infusion period than did control animals. The relation between the total amount of Ringer-Locke infused and the urine passed is not linear, but logarithmic, as is shown in Fig. 1(B), where the log urine volume/kg./5 hours is plotted against the total amount of Ringer-Locke infused/kg./5 hours. The relations depicted in Fig. 1(B) may be summarized by deriving two linear regression equations.

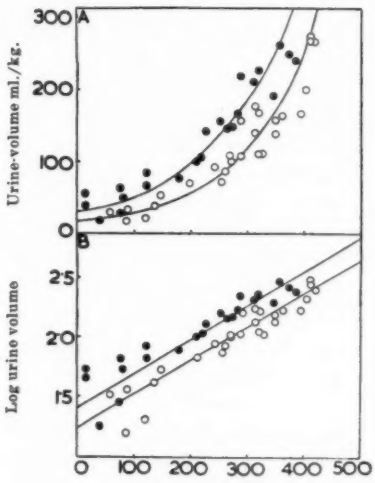

Fig. 1.

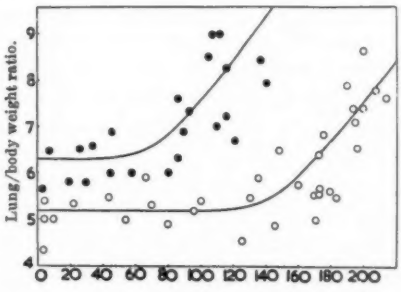

Fig. 2.

Fig. 1. (A) Relation between volume of Ringer-Locke infused (ml./kg./5 hours) and urine secreted (ml./kg./5 hours).
(B) Relation between volume of Ringer-Locke infused (ml./kg./5 hours) and log urine volume (ml./kg./5 hours).
● Noradrenaline group. ○ Control group.

Fig. 2. Relation between lung/body weight ratio (gm./kg.) and total excess tissue fluid ml./kg./5 hours).
● Noradrenaline group. ○ Control group.

In the case of animals given noradrenaline,

$$\text{Log U.V.} = 2{\cdot}0094 + 0{\cdot}00286 \ (\text{V.I.} - 208) \quad . \quad . \quad . \quad (1)$$

and in the controls,

$$\text{Log U.V.} = 2{\cdot}0244 + 0{\cdot}00298 \ (\text{V.I.} - 277) \quad . \quad . \quad . \quad (2)$$

where U.V. is urine volume ml./kg./5 hours

V.I. is volume of Ringer-Locke infused ml./kg./5 hours.

The regression coefficients are not significantly different from one another, indicating that an equal increment of Ringer-Locke produced a proportional

increase in amount of urine secreted in both groups. The noradrenaline animals secreted significantly more urine for a given infusion of Ringer-Locke. Thus for an infusion of 250 ml./kg./5 hours the average amount of urine calculated from equations (1) and (2) is 135 ml./kg./5 hours in the noradrenaline group and only 88 ml./kg./5 hours in the control group, the probability of this difference occurring by chance being less than 0·001.

For a given infusion, the noradrenaline animals thus eliminated a greater proportion of fluid, and retained a smaller net amount to expand the plasma and extracellular fluid volumes.

The relation between net fluid retained and the lung-body weight ratio. In Fig. 2 the lung/body weight ratio (L/B ratio) has been plotted against the total excess fluid oedema/kg. (i.e. fluid infused—urine volume—increase in plasma volume) which accumulated in the tissues during the five hour period of intravenous infusion in individual experiments at various transfusion rates. The graph thus represents the proportion of fluid which accumulated in the lungs as compared with the rest of the body. It may be seen that even at very low net infusions the L/B ratio was significantly higher in the noradrenaline group (mean 6·3) than in the controls (mean 5·2). Increasing the net infusion resulted in an earlier rise in L/B ratio in the noradrenaline animals compared with the controls. Thus an L/B ratio of 7·5 was obtained for a net infusion volume of 100 ml./kg./5 hours in the noradrenaline animals, but required a net infusion volume of 200 ml./kg./5 hours in the control group. It should be noted that the slopes of the regression lines depicting the relation between L/B ratio and excess tissue fluid in the two groups are not significantly different.

TABLE 1.

Relation of lung/body weight ratio to microscopic lung appearance.

L/B ratio gm./kg.	Control group	Animals given noradrenaline 0·3 μg./kg./min.
4–5	Normal lung appearance	
5–6	Slight pulmonary congestion, occasional large patches of oedema	Considerable pulmonary vascular congestion
6–7	Pulmonary congestion and more generalized lung oedema	Severe pulmonary congestion with a few scattered patches of oedema
7–8	Severe pulmonary oedema with tracheal froth	Severe pulmonary congestion with larger patches of oedema
8–9	Severe pulmonary oedema with copious tracheal froth	Congestion with moderately severe pulmonary oedema + tracheal froth
9–10		Severe pulmonary oedema with copious tracheal froth

Significance of raised lung/body weight ratio in noradrenaline experiment. Whereas in control animals transfused with Ringer-Locke solution, a rise in the L/B ratio above its normal level of 5, signifies accumulation of pulmonary oedema (Courtice and Korner, 1952), this was not entirely the case in those animals transfused with Ringer-Locke solution containing noradrenaline. There the L/B ratio was raised even at very low net infusion rates, and this rise appeared to be largely due to engorgement of the lungs with blood. The microscopic appearance of the lungs at various L/B ratios are summarized in Table 1.

It may be seen that whilst a L/B ratio of about 6 in the control group signified the presence of some pulmonary congestion with moderate patches of pulmonary oedema, in the noradrenaline group there was severe congestion with very little oedema. In general the noradrenaline animals had a grade of oedema corresponding to the L/B ratio —1 in the control group, and they always showed a greater degree of pulmonary congestion. In spite of this it may be seen from Fig. 2 that the accumulation of actual pulmonary oedema commenced at lower net transfusion rates in the noradrenaline group compared with the control animals.

TABLE 2.

The distribution of retained fluid at a net transfusion volume of 100 ml./kg./5 hours.

	Noradrenaline group	Control group
Total net infusion (ml./kg./5 hr.)	100	100
L/B ratio	7·5	5·2
Amount of oedema fluid in lung (ml./kg.)	1·4	very little
Amount of excess blood in lung (ml./kg.)	1·1	0·2
Increase in plasma volume (ml./kg.)	7·2	5·0
Serous effusion (ml./kg.)	0·8	3·5
Systemic extracellular fluid (ml./kg.)	90·6	91·3

Mean figures are given throughout; net transfusion volume equals total volume infused less urine secreted.

From the data depicted in Fig. 2 one may calculate roughly the percentage increase in pulmonary blood volume which occurred due to infusion of noradrenaline. Thus at net transfusion rates below 50 ml./kg./5 hours, that is before much pulmonary oedema begins to form in the noradrenaline group, the mean L/B weight ratio is $6{\cdot}34 \pm 0{\cdot}17$, and $5{\cdot}1 \pm 0{\cdot}12$ in the control groups, the difference being statistically significant ($P < 0{\cdot}001$). This difference of 1·2 gm. is largely due to an increased pulmonary blood volume in the noradrenaline group. Now the pulmonary blood volume in a rabbit may be assumed to be about 5 p.c. of its total blood volume (Wiggers, 1921). With an average blood volume of about 60 ml./kg. (Courtice and Gunton, 1949; Armin, Grant, Pels and Reeve, 1952), about 3 ml. would constitute the pulmonary blood volume in a normal rabbit. This would weigh approximately

3·5 gm. An increase of 1·2 gm. in the L/B ratio due to an increase in pulmonary blood volume as in the noradrenaline group thus implies an increase in pulmonary blood volume of 25–30 p.c.

The distribution of retained fluid in the body in the control and noradrenaline group has been summarized in Table 2. The results have been calculated for greater ease of comparison from various regression lines derived from the data. The results show that at that particular net infusion volume, there was a greater degree of pulmonary oedema in the lungs in the noradrenaline group, as well as a greater rise in the pulmonary blood volume. In addition there was a smaller amount of fluid in the serous cavities (peritoneum, pleura and pericardium) in the noradrenaline group than in the controls.

Increase in plasma volume in noradrenaline group and controls. The mean increase in plasma volume was 18±1·7 p.c. in the noradrenaline group, 24±3·1 p.c. in the unoperated controls and 17±3·2 p.c. in the operated controls. The expansion of the plasma volume by means of the Ringer-Locke infusion brings about a drop in plasma colloid osmotic pressure. This reduction in colloid osmotic pressure tends to diminish the reserve of pressure in the lungs for keeping the latter dry (Drinker, 1945; Cournand, 1950; Courtice, 1953). As the degree of plasma volume expansion was not significantly different in any of the above groups, the greater ease of inducing pulmonary oedema for a given net infusion volume in the noradrenaline group, does not depend on differences in the colloid osmotic pressure.

Cardiovascular changes during noradrenaline infusion.

Mean arterial pressure. The average rise in mean carotid arterial pressure in 20 animals during the five hour infusion period of noradrenaline at the rate of 0·3 μg./kg./minute was 25±2·9 mm. Hg. The blood pressure was well maintained throughout the five-hour period and showed no tendency to drop, apart from minor fluctuations. The actual final mean arterial pressure attained varied with the initial resting mean arterial pressure and the relationship may be summarized by the following regression equation:

$$\text{Final A.P.} = 127 + 0{\cdot}772\ (\text{initial A.P.} - 102) \quad . \quad . \quad . \quad (3)$$

S.E. of regression coefficient is 0·16

P is $<0{\cdot}001$.

Thus the lower the initial arterial pressure, the lower was the final blood pressure level attained.

In 11 operated controls the initial mean carotid pressure was 102±3·1 mm. Hg., whilst the final level was 102±2·8 mm. Hg. The infusion of Ringer-Locke alone had no apparent effect on the systemic blood pressure level.

Systemic venous pressure. Jugular venous pressures were measured in 20 animals given noradrenaline at 0·3 μg./kg./minute, and in 11 operated control animals.

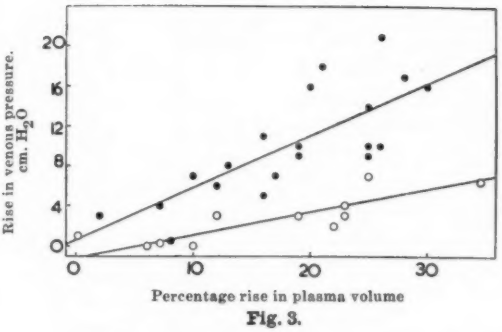

Fig. 3.

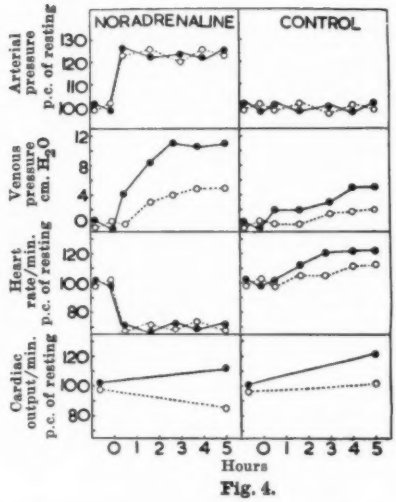

Fig. 4.

Fig. 3. Relation between percentage increase in plasma volume and rise in jugular venous pressure (cm. H_2O).

● Noradrenaline group. ○ Control group.

Fig. 4. Changes in mean arterial pressure (p.c. of resting), jugular venous pressure (cm. H_2O), heart rate/minute p.c. of resting), cardiac minute volume (p.c. of resting) in noradrenaline and control groups.

● Large infusion of Ringer-Locke. ○ Small infusion of Ringer-Locke.

Fig. 3 compares the relation between the rise in jugular venous pressures and the percentage rise in plasma volume in the noradrenaline group and in the operated controls. The jugular venous pressure was significantly higher in the noradrenaline group than in the control group, the difference in mean levels for a plasma volume increase of 18 p.c. being $7{\cdot}0 \pm 1{\cdot}0$ cm. water ($P < 0{\cdot}001$). The regression coefficients expressing the relation of venous pressure on increase in plasma volume also differ significantly. This means that a given increment of plasma volume produced a greater rise in venous pressure in the noradrenaline group than in controls.

Cardiac output. Cardiac output, arterial and jugular venous pressures and heart rates were determined on twelve rabbits. An initial resting value was obtained in each case, and six were subsequently given noradrenaline at the rate of $0{\cdot}3$ μg./kg./minute and six received infusions of Ringer-Locke solution only. The noradrenaline and control groups were further subdivided into groups of three animals, one group receiving an infusion at a rate giving very little pulmonary oedema, whilst the other received a transfusion at rates giving them fairly severe pulmonary oedema. The results are shown in Table 3.

TABLE 3.

Cardiovascular effects of infusion of noradrenaline.

	Resting cardiac output ml./kg./min.	Final cardiac output ml./kg./min.	Resting stroke volume ml./kg.	Final stroke volume ml./kg.	Resting heart rate/min.	Final heart rate/min.	Rise in venous pressure cm. H_2O	Resting O_2 consumption ml./kg./min.	Final O_2 consumption ml./kg./min.	Resting AV O_2 difference ml. p.c.	Final AV O_2 difference ml. p.c.	L/B ratio gm./kg.
Noradrenaline group												
(A)	309	304	1·20	1·70	252	170	0	13·5	14·3	4·35	4·70	5·6
	238	229	0·80	1·10	288	200	7	9·1	13·3	3·82	5·80	6·5
	253	169	0·82	0·88	306	190	7	10·6	13·3	4·21	7·88	6·0
Mean	267	234	0·94	1·23	282	187	4·6	11·1	13·6	4·12	6·12	6·0
(B)	208	252	0·63	1·00	306	249	15	10·8	15·8	5·18	6·25	7·6
	247	280	1·00	1·40	250	190	9	13·0	16·8	5·27	6·00	9·0
	230	242	0·83	1·20	270	200	10	14·8	15·1	6·55	6·20	7·0
Mean	228	253	0·82	1·20	275	213	11·3	12·8	15·9	5·66	6·15	7·9
Control group												
(A)	249	284	0·95	0·85	256	330	0	10·0	13·3	4·00	4·65	5·3
	183	191	0·63	0·63	300	294	3	11·4	12·1	6·23	6·34	5·5
	231	200	0·72	0·56	316	360	2	9·9	12·4	4·32	6·22	5·2
Mean	221	225	0·76	0·68	290	328	1·6	10·4	12·6	4·85	5·73	5·3
(B)	255	314	1·06	1·10	240	302	3	13·2	16·3	5·16	5·16	6·7
	237	309	0·80	0·93	290	336	8	10·8	15·0	4·53	4·85	7·6
	237	278	1·18	1·10	200	260	4	14·7	12·3	6·23	4·41	7·2
Mean	243	300	1·00	1·01	243	299	5·0	12·9	14·5	5·30	4·80	7·1

(A) = Transfusion giving minimal pulmonary oedema. (B) = Transfusion giving severe pulmonary oedema.

It may be seen that the resting values for both the noradrenaline and control groups were fairly comparable, signifying that both groups were in the same initial cardiovascular and respiratory state at the commencement of the experiment. Because of the rather large resting inter-animal variation, statistical analysis by means of the analysis of variance technique (Fisher, 1946) has been carried out on the percentage change after five hours from the resting value of some of the variables considered in Table 3.

Fig. 4 shows that the cardiac output rose to a greater extent from its initial resting value in both the control and noradrenaline subgroups receiving the more massive infusions, than in the animals receiving only small infusions. This difference was statistically significant ($P = 0{\cdot}01$). The cardiac output dropped on the average 14 p.c. from resting at the end of five hours in the noradrenaline group receiving small infusions and remained practically unchanged in the control group. At the end of five hours' more massive infusion the cardiac output had risen 13 p.c. from resting in the noradrenaline group, and 24 p.c. from resting in the control group. There was thus a tendency for the cardiac output to be slightly lower for a given infusion with noradrenaline, than for a control infusion. The present experiments, however, do not definitely establish this, as the probability of the difference in response between the noradrenaline and the control group at a given infusion rate was only 0·08, which is rather suggestive of a real difference.

There was a significant bradycardia with the noradrenaline infusions. The average drop in heart rate in the noradrenaline group was 29 p.c. ($P<0{\cdot}001$). In the control group there was a rise in heart rate following infusions. The average rise in heart rate was 18 p.c. in the control group ($P = 0{\cdot}001$). Mainly because of this difference in heart rates in the noradrenaline and control groups, the noradrenaline group showed a significant rise in stroke volume, whilst the control group showed little change.

The jugular venous pressure rose to a significantly greater extent in the noradrenaline group than in the controls, as was already noted in the larger transfusion series.

Systemic peripheral resistance calculated from the blood flow, arterial and venous pressure data, showed a significant increase in the noradrenaline group. The initial peripheral resistance was $0{\cdot}245 \pm 0{\cdot}016$ P.R.U. (Green, 1950) in the noradrenaline group and $0{\cdot}251 \pm 0{\cdot}015$ P.R.U. in the controls. At the end of the infusion period the peripheral resistance rose on an average by 25 p.c. of resting in the noradrenaline group and fell by 7·3 p.c. in the controls. The difference between the groups at the end of five hours was thus $32{\cdot}3 \pm 12{\cdot}9$ p.c. This is statistically significant ($P = 0{\cdot}03$). The rise in peripheral resistance in the noradrenaline experiment was thus roughly proportional to the rise in mean carotid arterial pressure (see equation 3).

The oxygen consumption/kg./minute was essentially similar in the two

groups. The initial mean resting oxygen consumption was 11·9 ml./kg./minute in the noradrenaline group and 11·7 ml./kg./minute in the controls. At the end of five hours it was 14·7 ml./kg./minute in the noradrenaline group and 13·9 ml./kg./minute in the controls. The discrepancy between the initial and final readings may have been partly due to a slight residual effect of the narcosis under which the operation was performed. In any case the experiments failed to demonstrate a significant calorigenic effect even following prolonged treatment with noradrenaline. There was an increase in ventilation following five hours' infusion in both groups. In the noradrenaline group the mean resting ventilation was 586 ml./kg./dry air at S.T.P., whilst at the end of five hours it was 766 ml./kg. dry air at S.T.P. In the control group the mean resting ventilation was 410 ml./kg. dry air at S.T.P. and 632 ml./kg. dry air at S.T.P. at the end of five hours. This change in ventilation was, however, quite physiological and unaccompanied by signs of overventilation which might invalidate cardiac output measurements by the Fick method. Thus in the noradrenaline group the mean initial R.Q. was 0·87 and became 0·91 at the end of five hours' transfusion, whilst in the control group the mean initial R.Q. was 0·77 and this changed to 0·82 at the end of the treatment period.

The arterial oxygen saturation only fell in those animals which showed a significant degree of pulmonary oedema. Thus in the noradrenaline animals with minimal pulmonary oedema the resting mean arterial oxygen saturation changed from 98 p.c. to 97 p.c. at the end of five hours, despite the raised L/B ratios due to an increase in pulmonary blood volume. In the noradrenaline group with significant pulmonary oedema the mean arterial oxygen saturation fell from 98 p.c. to 93 p.c. The corresponding figures for the control group was a change from 97 p.c. to 98 p.c. in the group with minimal oedema, whilst the saturation fell from 98 p.c. to 94 p.c. in the group with pulmonary oedema. The A.V. oxygen difference rose significantly from 4·89 resting to 6·12 volumes p.c. in the noradrenaline group but showed no significant change in the control group, the resting and five hour figures being 5·07 and 5·27 volumes p.c. respectively.

Effect of atropine on production of pulmonary oedema by infusions of noradrenaline.

It was felt that the considerable bradycardia accompanying the infusion of noradrenaline might partly account for the greater rise of venous pressure and pulmonary blood volume observed in the noradrenaline series compared with the control series. For this reason 4 rabbits were given an initial dose of 0·64 mg./kg. of atropine sulphate before commencing the infusion of noradrenaline. The noradrenaline drip was started five minutes after the atropine injection. The heart rate was measured at fifteen minute intervals and a further small dose of atropine was given if the heart rate showed any tendency to fall below the resting value. The results are summarized in Table 4.

TABLE 4.

Effect of atropine on production of pulmonary oedema following noradrenaline infusion.

	Number of animals	Dose of atropine mg./kg. 5 hrs.	Dose of noradrenaline µg./kg./min.	Initial blood pressure mm. Hg.	Final blood pressure mm. Hg.	Initial heart rate/min.	Final heart rate/min.	Rise in plasma volume p.c.	Rise in jugular venous pressure cm. H_2O	Total body oedema ml./kg./5 hr.	L/B ratio gm./kg.
Atropine group	4	0·71	0·3	107± 0·8	131± 3·8	322± 9·1	326± 8·9	7± 3	1·5± 0·6	95	7·6± 0·43
Noradrenaline group	20	—	0·3	102± 4·0	127± 0·8	280± 4·1	200± 3·0	18± 1·7	10± 1·2	85	7·0± 0·23
Operated control group	11	—	—	102± 3·1	102± 2·8	270± 7	318± 6·0	17± 3·2	2·7± 0·43	150	5·5± 0·3

TABLE 5.

Effect of haemorrhage on production of pulmonary oedema with noradrenaline infusions.

	Number of animals	Fluid infused ml./kg.	Urine passed ml./kg.	Blood pressure at start of 1st bleed mm. Hg.	Final blood pressure mm. Hg.	Rise in jugular venous pressure cm. H_2O	L/B ratio gm./kg.	Comment
Noradrenaline bled rabbits	5	75	10	128	<40 in 4 cases 80 in 1 case	9·0	7·2	Death within 60 minutes after 2nd bleed in four out of five cases
Control bled rabbits	3	110	45	98	98	6·5	5·4	All survived 5 hours

The atropinization abolished the noradrenaline-induced bradycardia. The rise in mean carotid blood pressure was in exact conformity with the rise obtained in the ordinary noradrenaline series (see equation 3). The jugular venous pressure rose by an average value of 1·5 cm. H_2O for an expansion in the plasma volume of 7 p.c. For this degree of plasma volume expansion the rise in venous pressure would be 4·3 cm. H_2O and 0·4 cm. H_2O in the noradrenaline and control experiments (see Fig. 3). The venous pressure obtained in the atropinized rabbits did not differ significantly from either of these values. Though atropinization did appear to lower the systemic venous pressure in rabbits given noradrenaline, it was not possible to say from the results whether it reduced it right down to the control level.

The average net infusion volume in the atropinized rabbits was 95 ml./kg./5 hours. All the rabbits had a considerable degree of pulmonary oedema, though the lungs did not appear to be quite as engorged with blood as in the ordinary noradrenaline experiments. For a net infusion volume of 95 ml./kg. the L/B ratio was 7·6 in the atropinized rabbits, 7·3 in the rabbits given noradrenaline only and 5·1 in control rabbits. The atropinized rabbits given noradrenaline thus had as great a degree of pulmonary oedema as the rabbits given noradrenaline alone, and this was significantly greater than in the case of the control rabbits.

Effect of haemorrhage on noradrenaline-induced pulmonary oedema.

Five rabbits were bled rapidly on two occasions in the course of noradrenaline infusions. The first bleeding occurred thirty minutes after commencing the infusion and the second bleeding one and a half hours later. Five ml. of blood were removed on each occasion, half the amount being taken from a catheter in the right auricle and the remainder from a catheter in the carotid artery. A similar procedure was carried out in 3 rabbits given Ringer-Locke solution alone.

Bleeding had little effect on the control rabbits. Their blood pressure became quickly restored to normal. They passed a normal amount of urine and the L/B ratio did not differ significantly from normal. The venous pressure was slightly greater at the end of five hours than normally. The rabbits infused with noradrenaline were markedly affected by the bleeding. After the first bleeding the blood pressure dropped gradually about 20 mm. Hg., and dropped further about half an hour after the second bleeding. Four out of five rabbits died within an hour after the second bleeding. Blood pressure dropped to very low levels in those animals despite continuing the noradrenaline infusion. The venous pressure was about at the same level as in the ordinary noradrenaline series. The L/B ratio was 7·2, slightly greater than in the ordinary noradrenaline series for a similar net infusion volume. Bleeding rabbits in the course of an infusion of noradrenaline, besides being lethal, certainly did not diminish the degree of pulmonary oedema.

Effect of large doses of noradrenaline.

In all experiments so far described the dose of noradrenaline given was 0·3 μg./kg./minute. In three experiments noradrenaline was administered in doses varying from 10–20 μg./kg./minute. The results were quite different qualitatively from those of the experiments with the smaller dose. The blood pressure rose by about 50–60 mm. Hg. for the first two hours, and then fell from this level of about 150 mm. Hg. to low levels of about 45–50 mm. Hg. in the course of the remaining three hours. The animals all had a considerable bradycardia, the heart rate falling from an average of 250 beats/minute to 120 beats/minute. The venous pressure rose very slowly and at the end of 5 hours averaged about 7 cm. of water. There was some haemoconcentration in all animals, the average initial haemoglobin being 91 p.c. and the final haemoglobin 112 p.c. The animals passed no urine at all during the five-hour period and received an average infusion of 130 ml./kg. The lungs of all the animals showed no trace of pulmonary oedema, but were very pale in colour; the mean L/B ratio was 4·7. All the animals had massive ascites, pleural and pericardial effusions; the mean total effusions/kg. was 15 ml./kg. and they were rather bloodstained. In these large doses infusions of noradrenaline did not lead to the development of pulmonary oedema.

DISCUSSION.

Cardiovascular action of noradrenaline.

Small doses of noradrenaline infused over a period of five hours produced systemic hypertension, bradycardia, an increased systemic venous pressure and a slight relative diminution in cardiac minute volume. There was also an increased pulmonary blood volume. It becomes important to decide whether the higher venous pressure and the raised pulmonary blood volume are the consequence of back pressure effects secondary to the systemic hypertension or are the result of vasomotor redistribution of blood. Redistribution changes in the circulatory system are indeed likely during prolonged arterial vasoconstriction, as the diminution in capacity of the arterial system must allow the accommodation of blood elsewhere; the most likely areas to which blood could be quickly moved are the low pressure systems in the circulation, viz. the systemic venous system and the pulmonary circulation (Hochrein and Matthes, 1933a and b).

In Fig. 5 the change in cardiac work from resting has been plotted against the different venous filling pressures in the animals receiving small and large amounts of Ringer-Locke containing 0·3 μg./kg./minute noradrenaline and in control animals. It may be seen that whilst the noradrenaline animals have a greater venous filling pressure the heart is not in a hypodynamic state

(Howarth, McMichael and Sharpey-Schafer, 1946; McMichael, 1952), as there is a proportional increase in cardiac work for a given change in filling pressure in both the controls and the noradrenaline group. We may conclude then that the systemic vasoconstriction does not stop the heart responding physiologically to meet the stress of increased circulatory load. There does not appear to be cardiac failure as defined recently by Cournand (1952).

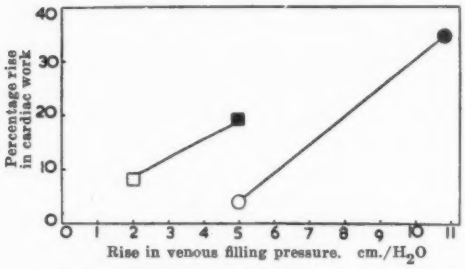

Fig. 5. Relation between average rise in venous filling pressure (cm. H_2O) and average increase in cardiac work (p.c. of resting value).

○ Noradrenaline group, small transfusion.
● Noradrenaline group, large transfusion.
□ Control group, small transfusion.
■ Control group, large transfusion.

The difference in slopes in the regression lines of the relation between venous pressure and expansion of plasma volume in Fig. 3 indicates that for a given increment of plasma volume the systemic venous system is nearer the end of its elastic limit in the noradrenaline experiments than in the controls. This could be due to a greater static blood volume in the venous system consequent upon blood redistribution or a venomotor effect of noradrenaline, the latter being rather unlikely in the light of the present findings with atropine.

The most suggestive indirect evidence that the greater ease of inducing pulmonary oedema with noradrenaline infusion does not depend on back pressure effects, but on the results of blood redistribution was given by the experiments during which rabbits were bled in the course of transfusion. Now this procedure reduces the rabbit blood volume, and although it almost certainly intensifies the noradrenaline-induced vasoconstriction, it nevertheless leads to a lowering of mean arterial pressure, but only a very temporary drop in jugular venous pressure. In the absence of marked interference with the coronary circulation, one would expect that if the rise in jugular pressure and raised pulmonary blood volume were due to back pressure effects of a heart labouring under a raised systemic arterial pressure, reduction in blood pressure and blood volume should ease the burden of the left ventricle and should thus lead to a diminution in pulmonary blood volume and systemic venous pressure; the animal should be able to carry on as well as the controls, who were not significantly affected by the bleeding. Instead of this the venous pressure rose more steeply soon after bleeding than in the ordinary noradrenaline series, and the animals developed pulmonary oedema even more easily than those of the latter series. Interference with the coronary circulation due to drop in blood pressure is unlikely to be the initiating factor in this sequence, as the maximum

fall in blood pressure only occurred terminally in the animals subjected to bleeding. It follows then that vasomotor redistribution of blood consequent on systemic vasoconstriction is probably of greater importance in explaining the raised pulmonary blood volume and venous pressure in the noradrenaline experiment. Intensification of vasoconstriction by haemorrhage in the course of noradrenaline infusion probably exaggerates the original redistribution of blood and thus accounts for the even greater ease in precipitating pulmonary oedema in these animals when compared with the ordinary noradrenaline series. The raised venous filling pressure in the noradrenaline experiments is probably a compensating mechanism (Howarth *et al.*, 1946; McMichael, 1952) to improve cardiac performance against moderate degrees of systemic hypertension.

The results of experiments with atropine together with noradrenaline suggest that the bradycardia does not play a big part in accounting for the greater ease in inducing pulmonary oedema during noradrenaline infusions. There was a suggestion that abolition of bradycardia tended to diminish the systemic venous pressure somewhat, though the series is too small to be definite on this point. The fact that pulmonary blood volume appears to be diminished to a negligible degree with atropine may probably be accounted for by the greater volume elasticity coefficient of the left atriovenous compared with the right atriovenous systems (Opdyke, Duomarco, Dillon, Schreiber, Little and Seely, 1948). Abolition of bradycardia might thus have a smaller effect in reducing pulmonary venous and left atrial pressures during infusion, than it has in reducing right auricular and jugular venous pressures. In any case abolition of the reflex bradycardia does not seem to alter significantly the pulmonary haemodynamics following noradrenaline infusions, as judged by the degree of pulmonary oedema.

The site of vasoconstriction rather than the actual blood pressure level appears to be very important in bringing about redistribution of blood to the systemic venous system and the pulmonary circulation. Thus in experiments with large doses of noradrenaline there was not apparently an increase in pulmonary blood volume and pulmonary oedema did not occur. Instead these animals had considerable effusions in their serous cavities. Daniel and Prichard (1951) have shown that intense sympathetic stimulation, or the injection of large doses of adrenaline, constricts the intra-hepatic branches of the portal tree in rats, rabbits, cats, dogs and monkeys, and this may explain the production of ascites with the large doses of noradrenaline. The fluid in the pleura probably comes initially from the peritoneal cavity and might accumulate owing to damming back from lymphatics caused by the rapid rate of lymph flow (Courtice and Steinbeck, 1950, 1951). The fact that the blood pressure was not sustained using these large doses of noradrenaline is probably due to this portal constriction, blood probably pooling in the splanchnic area drained by the portal tree.

Three lines of evidence then support the view that vasomotor redistribution of blood and not redistribution secondary to cardiac failure was important in the present experiment. The cardiac output and cardiac work increased proportionately for a given increment of venous pressure in the noradrenaline and control experiments. Bleeding intensified the degree of pulmonary oedema in animals given noradrenaline and had no effect on controls. The regional site of systemic vasoconstriction was more important than the actual hypertension as was seen in experiments with large doses of noradrenaline, where pulmonary oedema was not produced.

Little work has been done in man and animals on the detailed cardiovascular changes resulting from such prolonged infusions of noradrenaline. The present findings, however, are in good agreement with the results of short-term experiments in man carried out by Goldenberg *et al.* (1948). These workers also found a rise in mean pulmonary arterial pressure, which was not measured in the present study, but were unable to decide whether this was due to pulmonary arterial vasoconstriction or to back pressure effects. In the light of the present work, back pressure effects transmitted from the left ventricle are probably slight and the results of Goldenberg *et al.* suggest either pulmonary arterial constriction or a rise in pressure due to an increased pulmonary blood volume resulting from vasomotor blood redistribution. Blacket, Pickering and Wilson (1950) were unable to maintain a consistent hypertension for several days during noradrenaline infusions; the doses were much larger than those used here, and from their results it appears that the effect they obtained was a slightly lesser degree of that obtained in the present series with very large doses of noradrenaline (viz. portal vasoconstriction leading to pooling of blood in the splanchnic area). In the present series, doses of 0·3 μg./kg./minute noradrenaline, which mimic a physiological degree of sympathetic stimulation, consistently maintained an elevated blood pressure.

The cardiac output figures in the present experiment are in good agreement with findings previously reported from this laboratory (Courtice and Korner, 1952). In the present series, however, the heart rates of the rabbits were significantly greater than those of the earlier series. Perhaps the fact that the earlier series was carried out in winter and the second in summer may explain this difference.

Noradrenaline and transfusion.

The rabbits given noradrenaline at the rate of 0·3 μg./kg./minute secreted a significantly greater proportion of urine than control animals, whilst the animals given large doses of the drug secreted no urine. The first group secreted significantly more urine than could be accounted for by the rise in mean arterial pressure. Moyer and Handley (1952) found that in dogs given noradrenaline infusions there was first of all an increase in filtration fraction

with a diminution in renal plasma flow, and that as the dose was increased further both glomerular filtration and renal plasma flow decreased greatly and proportionately. Small doses of noradrenaline such as those used in the present experiments apparently produce efferent glomerular arteriolar constriction whilst afferent glomerular arteriolar constriction becomes more prominent with large doses.

The distribution of the retained fluid was different in the animals given small doses of noradrenaline than in control animals. In particular pulmonary oedema commenced to accumulate with smaller net infusion volumes in the noradrenaline series than in the controls. As the slopes of the regression lines in Fig. 2 depicting the relationship between oedema accumulating in the lungs and total excess tissue fluid was not significantly different in the noradrenaline and control series, selective permeability effects in the lung capillaries play no part in the experiment (see Courtice and Korner, 1952). These authors discussed fully the conditions under which fluid transfused would leave the pulmonary capillaries more readily. In the present experiments the large increase in pulmonary blood volume which occurs owing to noradrenaline infusion, causes an increase in the pulmonary vascular bed, and as a result less fluid is required to raise the capillary pressure sufficiently to overcome the osmotic pressure reserve and thus lead to filtration and accumulation of pulmonary oedema.

In the experiments of Courtice and Korner (1952) on anoxia where there was also undoubtedly an increase in pulmonary capillary blood volume, the L/B ratio in rabbits given very low infusion rates was not significantly increased from normal. The difference between this finding and the results of the noradrenaline experiments where the L/B ratio was raised even at very low infusion rates probably depends on the fact that the cardiac output was very low in the anoxia experiments but was nearly normal in the noradrenaline experiments. The interdependence of blood flow to actual amount of blood present in the lung has recently been stressed by Aviado, Cerletti, Alanis, Bulle and Schmidt (1952).

Related types of experimental pulmonary oedema.

A great deal of interest has been aroused in the production of pulmonary oedema by means of large rapid injections of adrenaline. Luisada (1928) precipitated this in rabbits, and found that the effects were diminished by the exhibition of narcotics. The doses used by him were very large and outside the physiological range. Whilst he postulated a central nervous causation of his pulmonary oedema, it seems likely that the true explanation of this type of oedema is haemodynamic. However, because of the rapidity of action and the large rises of blood pressure obtained, the cause of the oedema may have been back pressure from left ventricular failure rather than the explanations put

forward for the more moderate vasoconstriction due to noradrenaline (Johnson, Hamilton, Katz and Weinstein, 1937). Sarnoff and Sarnoff (1952) and Sarnoff and Berglund (1952) have suggested redistribution of blood following systemic vasoconstriction resulting from the intracisternal injection of fibrin (Cameron and De, 1949).

In the light of the present experiments it appears then that physiological degrees of systemic vasoconstriction raise the pulmonary blood volume, this being the result of vasomotor blood redistribution, not apparently due to cardiac failure with back pressure effects. The rise in pulmonary blood volume with physiological degrees of vasoconstriction in normal animals is not sufficient to induce pulmonary oedema *per se*, but renders the animals more susceptible to circulatory overloading by means of Ringer-Locke solutions. It is easy to see that in pathological states where the pulmonary blood volume may be initially high, even a mild vasoconstrictive episode of the extent mimicked in the present experiments might well elicit a bout of clinical pulmonary oedema; and this may be brought about by physiological adjustment mechanisms unaccompanied by aggravation of any existing clinical cardiac failure.

SUMMARY.

In unanaesthetized rabbits five hours' infusion of noradrenaline at the rate of 0·3 μg./kg./minute produced systemic hypertension, reflex bradycardia abolished by atropine, a rise in systemic venous pressure, an increase in pulmonary blood volume and no significant change in cardiac output.

The increase in pulmonary blood volume was thought to account for the increased susceptibility to pulmonary oedema of rabbits transfused with Ringer-Locke solution containing noradrenaline, compared with controls given Ringer-Locke alone. The greater susceptibility to pulmonary oedema was not abolished by atropine, or by reducing the total blood volume by bleeding the rabbits.

Rabbits given small doses of noradrenaline secreted more urine for a given infusion volume of Ringer-Locke solution than control rabbits. This was believed to be due to efferent glomerular arteriolar constriction.

With doses of noradrenaline from 10–20 μg./kg./minute infusion of Ringer-Locke solution did not lead to the development of pulmonary oedema, but resulted in the production of massive serous effusions.

It was thought that the rise in systemic venous pressure and increased pulmonary blood volume was the result of vasomotor redistribution of blood and was not due to cardiac failure.

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